University of Alberta

Regulation and Molecular Mechanisms of Stratifin-Induced MMP-1 Expression in Fibroblasts

by

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DEDICATION

To my Father and Mother:
Dr. Rev. George Lam and Mrs. Grace Lam,
without whom I would not be here.

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LIST OF ABBREVIATIONS

AP-1	activator protein-1
ATF-2	activating transcription factor-2
ATX	autotaxin
BSA	bovine serum albumin
CRE	cyclic AMP responsive elements
DMEM	Dulbecco's Modified Eagle Media
ECM	extracellular matrix
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
bFGF	basic fibroblast growth factor
GDP	guanosine diphosphate
GITC	guanidinium isothiocyanate
Glu	glutamine
Gly	glycine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTP	guanosine triphosphate
HSc	hypertrophic scarring
HSP-27	heat shock protein-27
IGF-1	insulin-like growth factor-1
IL-1	interleukin-1
JNK	c-Jun N-terminal kinase
KCM	keratinocyte-conditioned medium
KDAF	Keratinocyte-Derived Antifibrogenic Factor
KGF	keratinocyte growth factor
KSFM	Keratinocyte Serum-Free Media
LPA	lyso-phosphatidic acid
Lyso-PLD	lyso-phospholipase D
MAPK	mitogen-activated protein kinase
MAPKAP-K	MAPK-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MEF2	myocyte-enhancer factor 2
MEK	MAPK/ERK kinase
MMPs	matrix metalloproteinases
MMP-1	matrix metalloproteinase-1, collagenase
MNK	MAPK-interacting kinase-1
mRNA	messenger ribonucleic acid

PBS	phosphate-buffered saline
PD	PD098059
PDGF	platelet-derived growth factor
PGE2	prostaglandin E2
Pro	proline
S1P	sphingosine-1-phosphate
SAPK	stress-activated protein kinase
SB	SB203580
Ser	serine
Sos	son of sevenless
SP	SP600125
SRE	serum response element
SRF	serum response factor
TCF	ternary complex factor
TGF-β	transforming growth factor-β
Thr	threonine
TNF	tumor necrosis factor
Tyr	tyrosine
VEGF	vascular endothelial growth factor

CHAPTER 1:

GENERAL INTRODUCTION

1.1 Introduction

The primary function of the skin is to serve as a protective barrier against the environment. Loss of the integrity of large portions of the skin as a result of injury or illness may lead to major disability or even death. Every year, more than 1.25 million people in the United States have burns (1) and 6.5 million have chronic skin ulcers caused by pressure, venous stasis, or diabetes mellitus (2). However, recent advances in cellular and molecular biology have greatly expanded our understanding of the biologic processes involved in wound repair and tissue regeneration (3).

Wound healing is a dynamic process accomplished through complex interactions between stimulatory and regulatory cytokines and growth factors and fibroblasts, epithelial cells, endothelial cells and extracellular matrix (ECM) components. During the process of wound healing, a sequence of inflammation, tissue repair and reorganization results in the formation of mature scar tissue. The outcome of burn wounds can be normotrophic healing or abnormal wound healing, resulting in chronic ulcers or hypertrophic scars and keloids.

Traditional teaching has suggested macrophages as the orchestrators of this process, because they are a source for stimulatory cytokines (4). However, there is increasing evidence for paracine and autocrine cytokine interactions between other types of skin cells. These interactions are also likely to be important in wound healing. Specific interactions between keratinocytes and dermal fibroblasts are associated with effective and efficient wound healing. Keratinocytes produce mediators, which affect fibroblast function (5-8). Thus, it is likely that keratinocytes may have significant effects on fibroblast wound healing activities.

We have recently isolated a keratinocyte-derived factor from keratinocyte-conditioned medium and subsequently identified it as the extracellular form of stratifin, or 14-3-3 σ, with potent MMP-1 stimulatory effects in dermal fibroblasts. Although the nature of stratifin has been determined and its collagenase stimulatory effect validated, the question of its molecular mechanism by which it signals collagenase induction as well as the regulation of its efficacy in dermal fibroblasts have yet to be determined.

Chapter 2 provides a detailed review of literature relevant to the subsequent chapters. Chapters 3 and 4 describe the methology, results and conclusions of the experiments pertaining to insulin regulation of stratifin and the molecular mechanism by which stratifin induces MMP-1 in fibroblasts, respectively. Chapter 5 provides general discussion and conclusions from the data described in the previous chapters and provide suggestions for the direction of future studies.

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CHAPTER 2:

LITERATURE REVIEW

2.1 WOUND HEALING PROCESS

2.1.1 Introduction

Normal wound healing consists of an intricate cascade of specific overlapping events. These vital sequential stages in the wound healing process are namely inflammation, fibroplasia, angiogenesis, epithelialization, extracellular matrix (ECM) deposition, remodeling, and scar maturation. In recent years, a plethora of studies has identified and characterized major cytokines and growth factors involved in wound healing. Originally described for their ability to influence cell growth, these factors are now recognized to have a vast array of activities including chemotaxis, cytoskeletal structure, gene expression and pro-inflammatory effects.

2.1.2 Inflammatory Phase

Skin repair begins with the formation of a fibrin-rich blood clot, which provides a provisional matrix for subsequent reparative events to follow. The formation of the fibrin clot is initiated by degranulation and coagulation of platelets. Platelet adhesion and aggregation are critical to hemostasis. Several adhesive proteins are released into the injured site, which include fibrinogen, fibronectin, thrombospondin, and van Willebrand factor. The first three factors are essential for proper platelet aggregation, and von Willebrand factor mediates platelet adhesion to fibrillar collagens through interaction with platelet membrane glycoprotein receptor

GIb. Activated platelets not only aggregate and trigger blood coagulation to effect hemostasis in disrupted blood vessels but also they release many growth factors including epidermal growth factor (EGF) (1), insulin-like growth factor-1 (IGF-1) (2), platelet-derived growth factor (PDGF) (3, 4), and transforming growth factor-β (TGF-β) (5). These cytokines promote cell migration and growth by recruiting neutrophils, macrophages, epithelial cells, mast cells, endothelial cells, and fibroblasts into the site of injury.

Neutrophils are the predominant cell type in the wound within 24 hours after injury. The major function of the neutrophil is to remove foreign material, bacteria, and devitalized tissue by the process of phagocytosis (6). Following hemostasis, monocytes are drawn to the wound bed by chemotactic effect of low concentrations of TGF-β. Once they arrive at the wound site, monocytes are converted to macrophages through subsequent exposure to high concentrations of TGF-β (7). Macrophages then infiltrate the wound to remove damaged or nonfunctional host cells and foreign debris while releasing chemotactic factors such as PDGF and TGF-β for the proliferation and migration of fibroblasts. The influx of monocytes/macrophages is critical in tissue repair since studies have demonstrated that macrophage-depleted animals exhibited delayed fibrosis and wound debridement (8).

2.1.3 Proliferation and Angiogenesis

Migration and proliferation of endothelial cells and mast cells occurs as neovascularization is needed to provide oxygen and nutrients for continued growth.

Angiogenesis is characterized by capillary buds sprouting from blood vessels adjacent to the wound itself. It is initiated by basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), both of which promote angiogenesis by stimulating endothelial cell proliferation leading to the formation of capillary tubes. Endothelial cells also release plasminogen activator and procollagenase in response to bFGF stimulation. Plasminogen activator converts plasminogen and procollagenase to plasmin and collagenase, respectively, both of which serve to digest the basement membrane (9). Stimulation of angiogenesis is also caused by the release of heparin by mast cells (10).

Re-epithelialization begins with the movement of epithelial cells from the free edges of the wound as well as hair follicles, sebaceous and sweat glands (11). Epidermal growth factor (EGF) stimulates migration of epithelial cells to reestablish the integrity of the epidermis as a functional barrier to the harsh external environment (12). If the basement membrane is disrupted post-injury, epithelial cells migrate over a provisional matrix of fibrin and fibronectin. If the basement membrane is still intact, however, the hemidesmosomes that link epithelial cells to basement membrane are temporarily disrupted during cell migration. Once re-epithelialization has occurred, epithelial cells restore the basement membrane by producing laminin and type IV collagen (13).

2.1.4 Matrix Deposition

Fibroblast proliferation is required for the formation of new granulation tissue, which develops from the connective tissue in the damaged area and consists mainly of small vessels, inflammatory cells, fibroblasts, myofibroblasts loosely placed within ECM proteins (14). Fibroblasts, which become the predominant cell type in the wound, begin to produce the new matrix needed to restore structure and function to the tissue (14). These cells arise from the vicinity of uninjured tissue and nearby vessels in response to platelet, leukocytes, and macrophage-released chemoattractants such as TGF-β. When attached to the provisional fibrin matrix, fibroblasts begin to produce extracellular matrix components such as fibronectin, hyaluronic acid, proteoglycans and most importantly collagen, which is the primary structural component of repaired tissue (15, 16). Hyaluronic acid is the predominant glycosaminoglycan present early in healing wounds, whereas proteoglycans containing chondroitin sulfate and dermatan sulfate appear several days later (17). As the matrix matures, fibronectin and hyaluronic acid disappears and collagen bundles grow in order to increase tensile strength in wound site. Since type III collagen is deposited prior to type I (18), the former may form the template for the deposition of the latter, which is a stronger and thicker collagen fibril.

2.1.5 Wound Remodeling

The final phase of wound healing is the transformation of granulation tissue into mature scar, characterized by a systematic regression of granulation tissue, gradual loss of cellularity and vasculature as well as restructuring of the extracellular matrix. This process requires a delicate balance between matrix biosynthesis and degradation. The degradation of extracellular matrix occurs through the action of collagenase, proteoglycanases, and other proteases released from mast cells, macrophages, endothelial cells and fibroblasts. In contrast, the ECM biosynthesis is mainly the function of dermal fibroblasts, which produce collagens, fibronectin and proteoglycans. The granulation tissue is progressively replaced with more organized and elastic ECM (19). Any aberration to this balance by either an excess in ECM synthesis or a deficiency in degradation or remodeling may result in the formation of keloids or hypertrophic scarring (HSc) (20, 21). Thus, the remodeling phase is characterized by equilibrium between collagen synthesis and collagen degradation in effort to re-establish the connective tissue matrix.

2.2 ABNORMAL WOUND HEALING

2.2.1 Impaired Wound Healing

The dynamic interactions and feedback control mechanisms among the various components in the different stages of wound healing govern the direction of the repair. Thus, any aberration in the fundamental process of wound healing can result in poorly healing chronic wounds at one extreme and excessively healing hypertrophic scars and keloids at the other (22). Diabetic ulcers are an excellent example of how multiple physiologic and biochemical defects can lead to impaired healing. Diabetic ulcers are also prone to infection because of impaired granulocytic function and chemotaxis (23). Other abnormalities associated with diabetic ulcers include prolonged imflammation, impaired neovascularization, decreased synthesis of collagen, increased levels of proteinases, and defective macrophage function (24, 25).

2.2.2 Fibroproliferative Disorders

Hypertrophic scarring (HSc) and keloids are unique human dermal fibroproliferative disorders characterized by excess extracellular matrix (ECM) deposition in the dermis and subcutaneous tissues. Unlike normal wound repair that results in a fine-line scar, HSc and keloids form hard, elevated, red, and sometimes itchy scars that may cause pain, disfigurement, and contractures. The development of contractures is by definition the pathological shortening of scar tissue, which result in cosmetic and functional deformity, as opposed to wound contraction in normal healing, which acts to reduce the wound surface area. While HSc remains within the confines of the wound margin and eventually undergoes at least partial spontaneous resolution, keloids are able to enlarge and extend beyond the original wound margin and thus capable of invading surrounding tissue. Moreover, keloids usually require medical intervention as they rarely regress spontaneously.

Keloids and HSc contain large, thick collagen fibers composed of numerous fibrils closely packed together in which the collagen fibers appear more irregular than in normal dermis (26). HSc and keloid scars are characterized by an increased amount of dermal collagen (27), proteoglycans (28, 29), fibronectin (30, 31), and tissue water (32). Moreover, in HSc and keloid conditions, abnormalities in cell migration and proliferation, inflammation, synthesis and secretion of extracellular-matrix proteins and cytokines, and remodeling of the wound matrix have all been described (33). Increased activity of fibrogenic cytokines (eg. transforming growth factor β1, insulin-like growth factor 1, and interleukin-1) and exaggerated responses to cytokines have been noted (34, 35). In addition, abnormal epidermal-mesenchymal interactions and mutations in regulatory genes (such as p53) have recently been proposed to help explain abnormal healing (36, 37).

The development of HSc appears to occur following a prolonged period of inflammation. Prolongation of the inflammatory stage of wound healing due to infection or an extremely large or deep wound can lead to an exaggerated response by inflammatory cells and a subsequent excess of fibrogenic cytokine release of TGF-β and IGF-1 (9). Deitch et al (1983) indicated if the burn wound healed between 14 and

21 days then one third of the anatomic sites became hypertrophic; if the burn wound healed after 21 days then 78% of the burn sites developed hypertrophic scars. In addition, several areas including the chest, upper extremity, and foot were especially likely to become hypertrophic. In contrast, the hands, face, and neck region were more likely to heal without wound problems than other anatomic areas (38). The concept of wound tension has been used to explain the increased incidence of hypertrophic scar and keloid formation in areas such as the presternal region of the chest, the back, and the deltoid region. Also, it appears that in keloids the phases of fibroplasia and collagen maturation of wound healing become extremely prolonged. Therefore, an important indicator of whether HSc and keloids would develop is the time required for the burn to heal.

2.2.3 Aberrations of Extracellular Matrix Components

Excessive biosynthesis of ECM proteins by fibroblasts has been proposed as one of the potential contributing factors to the accumulation of excessive matrix in keloids and HSc. This excessive collagen accumulation is the result of selective increased collagen synthesis compared to total protein synthesis as demonstrated both in keloid biopsies (39, 40) and in early passage keloid fibroblasts compared to normal skin or normal scar fibroblasts. Fibroblasts isolated from keloids produce about 2 to 3 times more collagen than fibroblasts isolated from normal skin of the same patients (41). Moreover, Ghahary et al. (1992) have found after analysis of many pairs of HSc and normal fibroblasts that about half of the HSc strains produced significantly more collagen in vitro, and mRNA for proα2[I] chain of type I collagen was increased in five of six strains compared with normal skin fibroblasts. Messenger RNA extracted directly from HSc tissue revealed an increase in proα2[I] chain of type I collagen and proα1[III] chains of type III. (42).

Excessive matrix accumulation in fibroproliferative disorders can occur not only by increased synthesis of ECM proteins but also by a reduction in matrix degradation, either intracellularly or extracellularly. Many HSc fibroblasts have demonstrated reduced mRNA for collagenase as well as net reductions in ability to digest soluble collagen compared with their normal fibroblast pairs. These findings resemble scleroderma fibroblasts, which also lack collagenase activity, suggesting that this feature of HSc fibroblasts is a frequent and consistent finding that is a significant factor in dermal fibroproliferative disorders (43). Activated fibroblasts in HSc and keloids are unable to degrade collagen, which may inhibit their ability to remodel the pre-existing, randomly oriented collagen into a more uniform, organized matrix.

2.2.4 Dermal-Epidermal Interaction

Dermal-epidermal interaction has been the subject of several recent studies. Unhealed burn wounds lack an epidermis, suggesting a possible regulatory role for the epidermis in controlling dermal fibroblast matrix synthesis. It has been shown that cultured epidermal cells used as allografts for burn wounds allow rapid epithelialization (44). In addition, cell lysates from cultured keratinocytes stimulate and accelerate wound healing by 8 days when compared with treated and control groups (45). Further studies demonstrate that lysates of cultured keratinocytes contain mitogenic activity for keratinocytes, endothelial cells, and fibroblasts. Treatment with lysates of keratinocyte cultures caused two-fold stimulation of epithelialization (46). Proliferation of dermal fibroblasts and matrix modulation in response to keratinocyte-conditioned medium (KCM) has also been studied. The results revealed that KCM significantly increases fibroblast replication and decreases collagen synthesis. This finding suggested that synthesis of collagen by dermal fibroblasts appears regulated by some soluble keratinocyte products (47).

As hypertrophic scars most commonly occur when epithelialization has been delayed during, for example, the healing of deep dermal burn wounds, studies have proposed that cellular mechanisms in the pathogenesis of hypertrophic scarring are more complex than isolated dermal phenomena and result from abnormalities in the epidermal-dermal crosstalk (48). A recent study shows that keratinocytes derived from normal skin and hypertrophic scar differ in their effects on the proliferation and collagen synthesis of hypertrophic scar fibroblasts. Supernatant from normal skin keratinocytes inhibited HSc fibroblast proliferation and collagen secretion but increased collagen synthesis. On the contrary, HSc supernatant increased collagen synthesis and secretion with no effects on HSc fibroblast proliferation (49).

During wound healing, keratinocytes migrate over the wound bed, an activity associated with lysis of basement membranes, and hence requiring the presence of matrix metalloproteinases (MMPs). MMPs are also required for the production and remodeling of the granulation tissue (50). Keratinocyte-conditioned medium also stimulates the expression of MMP-4 (72 and 92 kDa) in human keratinocytes and fibroblasts (72 kDa). This finding suggests that an autocrine/paracrine control mechanism is involved in MMP production in these cell types during wound healing. It has also been suggested that stimulation of wound healing seen in wounds receiving sheets of keratinocytes is partly due to expression of MMPs (50). Recent studies have shown KCM stimulating a 5.7-fold increase in the level of MMP-1 and also inducing a 1.8-fold increase in fibroblast proliferation (51). In fibroblasts exposed to KCM, mitogen-activated protein kinase (MAPK) signaling through both the extracellular signal-related kinase pathway and p38 pathway occurred. This suggests that keratinocyte-fibroblast interactions are mediated by multiple stimulating agents acting on specific receptors to induce signaling through different MAPK pathways and thus jointly altering key biological functions.

Although the presence of some factors released from or associated with keratinocytes with wound healing abilities have been appreciated in some studies (52, 53), no specific factor(s) in either cell lysate or keratinocyte-conditioned medium has been identified in previous studies as fully responsible for accelerated wound healing and epithelialization in treated animals. Silver et al (1988) evaluated the possible regulatory role of epidermal cell extract(s) on keratinocytes and fibroblasts. It was found that cell extracts of cultured human keratinocytes stimulated replication of epidermal cells and their migration from wound edges and remnants of hair follicles and sebaceous glands but at the same time modulated contraction of collagen matrix and suppressed fibroblast proliferation (53). In a keratinocyte/fibroblast co-culture system, Goulet et al. (1996), reported a cellular cooperation between human keratinocytes and dermal fibroblasts resulting in increased DNA synthesis by keratinocytes through secretion of some soluble factors in the culture medium. Moreover, epidermal cell growth and metabolic activities were up-regulated in co-culture compared to the control (54).

Recently, El-Ghalbzouri et al (2002) noted that in the presence of fibroblasts, not only was keratinocyte proliferation increased, but also the epidermal morphology was improved. With increasing numbers of fibroblasts, the expression of markers associated with keratinocyte activation, e.g. keratins 6, 16, 17 and the cornified envelope precursor SKALP decreased, and involucrin localization shifted toward the granulosum layer. As well, expression of integrin subunits $\alpha 2$, $\alpha 3$, and $\beta 1$ was up-regulated, indicating keratinocyte activation (55).

Another cross-talk mediator between keratinocyte and fibroblasts is keratinocyte growth factor (KGF). KGF is produced by dermal fibroblasts and diffuses into the epidermis where it stimulates keratinocyte proliferation. KGF is dramatically overexpressed during wound healing and enhances wound reepithelialization, suggesting its importance in tissue repair (56-58). Prostaglandin E2 (PGE2), involved in proliferation and differentiation of keratinocytes, is another factor synergistically enhanced in fibroblasts by the co-culture system. In individual monolayer cultures of keratinocytes or fibroblasts, PGE2 production was negligible but was greatly enhanced by keratinocyte-conditioned medium. The enhanced production of PGE2 was due to induction of COX-2 mRNA mediated by a precursor of interleukin-1 alpha released from keratinocytes (59).

TGF-β is a multi-potent growth factor in tissue homeostasis regulating cell proliferation, adhesion, migration and differentiation as well as extracellular matrix deposition. Le Poole et al (1999) demonstrated suppression of fibroblast TGF-β mRNA and protein expression in the presence of keratinocytes. The authors suggest that expression of TGF-β by fibroblasts is down-regulated by paracine actions of some factor(s) secreted by keratinocytes in healing skin (60).

Sauder et al. (1990) demonstrated that interleukin-1 (IL-1) enhances epidermal wound healing. Interleukin-1, which is synthesized by keratinocytes in response to injury, has been demonstrated to stimulate fibroblast and keratinocyte growth, collagen synthesis by fibroblasts, and chemotaxis of keratinocytes. Recently, IL-1 has been shown to regulate keratinocyte proliferation and differentiation in a fibroblast-mediated paracrine manner by modulation of KGF and granulocyte-macrophage colony-stimulating factor (GM-CSF) in fibroblasts. Both KGF and GM-CSF are antagonistically regulated by two AP-1 subunits, c-Jun and JunB, which are transcription factors that mediate gene regulation in response to extracellular stimuli (61). In a pig model, topical administration of dermal cream containing IL-1α also significantly enhanced wound healing (62). However, no attempt has been made to differentiate IL-1α induced acceleration of wound healing from other factors that might be released from keratinocytes, either *in vitro* or *in vivo*. It is also not clear which of these biological activities is related to any of the previously identified growth factors and cytokines released from keratinocytes.

2.3 The 14-3-3 protein family

2.3.1 Introduction

The 14-3-3 protein family was originally identified in 1967 by Moore and Perez during a systematic classification of brain proteins (63). The name '14-3-3' is derived from the combination of its fraction number on DEAE-cellulose chromatography and its migration position in the subsequent starch-gel electrophoresis. In humans, there are seven distinct 14-3-3 isoforms (β, γ, ε, σ, ζ, τ and η) each encoded by a distinct gene (64, 65). The phosphorylated forms of β and γ were initially described as α and δ, respectively (66). The 14-3-3 proteins are present within all eukaryotic cells and exist mainly as homo- and heterodimers with monomeric molecular mass of approximately 30 kDa and an acidic isoelectric point of 4-5 (67). The first function ascribed to this family of proteins was activation of tyrosine and tryptophan hydroxylases (64). Since then, 14-3-3 has emerged as a group of multifunctional proteins that bind to and modulate a wide array of cellular proteins involved in processes such as metabolism, signal transduction, cell-cycle control, apoptosis, cytoskeletal structure, protein trafficking, transcription, stress responses, and malignant transformation.

2.3.2 14-3-3 Structure

Crystal structures of 14-3-3 various isoforms have been determined (68, 69) and reveal that they are highly helical, dimeric proteins. Each monomer is composed of 9 antiparallel α-helices (denoted αA-αI) with the dimer interface formed from helices αA, αC, and αD (Figure 2.6.1). Despite their genetic diversity, 14-3-3 isoforms are highly homologous proteins with large amounts of sequence identity and conservation (70-72) (Figure 2.6.2). There are several conserved regions which form the dimer interface as well as line the central ligand binding channel of the dimeric 14-3-3 molecule including many of the residues that form direct ligand contacts (71). The dimeric structure of 14-3-3 creates a large negatively charged channel. The invariable regions of all the isoforms are mainly found lining the interior of this channel, while the variable residues are located on the surface of the protein. The residues involved in dimerisation are 5-21 in the A-helix of one subunit and residues 58-89 of the C and D helices of the other.

The dimeric structure of the 14-3-3 protein allows it to bind two ligands simultaneously. Previous studies have suggested that 14-3-3 proteins may serve as a novel type of 'adapter proteins', mediating interactions from different proteins on each subunit of the dimer. For example, the ability of diverse members of this family to form heterodimers between ε and ζ and between τ and ζ isoforms (73) may allow the interaction between signaling proteins that do not associate directly with each other. Other evidence has been provided that 14-3-3 can mediate the association of Raf-1 with Bcr (74) or A20 (75); however, the physiological significance of these complexes is unclear.

It was shown that 14-3-3 contains phospho-peptide binding residues that are conserved within all isoforms. The binding site for the phosphoserine consists of a basic pocket composed of Lys-49, Arg-56 and Arg-127, as well as Tyr-128, within the C and E helices (76). As there are seven known mammalian isoforms of 14-3-3 that are all highly conserved, each isoform must have distinct functions. Binding to the variable surface of the protein could account for the observed specificity of interaction with diverse target proteins. For example, several isoforms of 14-3-3 are involved in the G2/M cell cycle checkpoint: 14-3-3 σ is responsible for sequestering the cdc2-cyclin B1 complex in the cytoplasm, while the β and ε isoforms bind Cdc25C (77).

2.3.3 Function of 14-3-3

To date, a plethora of functions have been ascribed to the 14-3-3 protein family. In brain tissue, 14-3-3 proteins were shown initially to stimulate the calmodulin kinase II-dependent activation of tyrosine and tryptophan hydroxylases during the synthesis of serotonin and dopamine (78). Subsequently, 14-3-3 proteins were demonstrated to modulate protein kinase C activity (65, 79). Since then, 14-3-3 proteins have been recognized to activate calcium-dependent exocytosis in permeabilized adrenal chromafin cells (80); stimulate exoenzyme S of *Pseudomonas aeruginosa* which ADP-ribosylates Ras and other host GTP-binding proteins (81); stimulate mitochondrial import in rat (82); function as part of the cell-cycle-control DNA-damage checkpoint in the fission yeast *S. pombe* (83); associate with polyomavirus middle tumor antigen (84); complex with c-Bcr and Bcr-Abl (85); bind and perhaps stimulate Raf activity (86-89); and complex with *cdc*25 phosphatase (90).

2.3.4 14-3-3 Target Binding

How the family of 14-3-3 proteins are able to interact with many different proteins and subsequently influence critical cellular pathways is a topic of intense interest. Studies performed by Muslin *et al* (1996) demonstrated the ability of 14-3-3 proteins to bind phosphoserine/phosphotheonine residues (91). Analyses of known 14-3-3 binding sites have defined two high-affinity phosphorylation-dependent binding motifs that are recognized by all 14-3-3 isotypes: RSXpSXP (mode 1) and RXXXpSXP (mode 2), where pS represents phosphoserine (71, 92). Experiments using phosphorylated peptides co-crystallised with 14-3-3 proteins have revealed that these motifs are recognized by a binding cleft within the conserved interior of the protein.

In contrast, some 14-3-3 interactions are independent of phosphorylation. Binding of 14-3-3 to exoenzyme S, p190RhoGEF and the R18 peptide inhibitor does not require a phosphorylated residue (93-97). Regardless of whether the interactions are dependent on phosphorylation or not, all targets interact with the same binding domain on 14-3-3 (96).

High-affinity 14-3-3-binding sites of various targets such as BAD, Raf-, and Cb1 as well as low-affinity 14-3-3-binding sites of cell-cycle regulator Cdc25B have been reported (92, 98). The high-affinity site on the target proteins is thought to act as a 'gatekeeper', recruiting the 14-3-3 dimer (99). Binding of a 14-3-3 monomer to this site could then allow other low-affinity sites to engage the second monomer, stabilizing the overall target-14-3-3 dimer complex. Furthermore, phosphopeptide containing two motifs binds 14-3-3 with a 30-fold greater affinity than a phosphopeptide containing one motif (92).

2.3.5 Regulation of Intracellular 14-3-3

2.3.5.1 Introduction

Several potential modes of 14-3-3 regulation are currently being explored, including phosphorylation regulation, subcellular localization, and isoform-specific expression in cells. Furthermore, a recent idea regarding a mechanism for 14-3-3 regulation suggests that association of 14-3-3 with intermediate filaments, such as vimentin, may serve to isolate 14-3-3 proteins and modulate their function by limiting their availability to other target proteins (100).

2.3.5.2 Phosphorylation Regulation

While the phosphorylation of some 14-3-3-binding motifs appears to be constitutive, other binding motifs are highly regulated by kinases that are activated under specific conditions. Identification of mode 1 and mode 2 binding motifs has advanced the discovery of many kinase regulators including sphingosine-dependent protein kinase (SDK1) (101, 102), casein kinase 1 (103), PKAs and PKCs (76).

Studies by Aitken (2002) reported that phosphorylation of specific 14-3-3 isoforms can modulate their binding (76). In 14-3-3 ζ, three phosphorylation sites have been determined: S58 (102), S184 (66), and T232 (103). S184 lies within a proline-directed kinase consensus sequence, S^{184}PEK, and is phosphorylated in both 14-3-3 ζ and β in brain tissue. As mentioned previously, the phosphorylated forms of 14-3-3 β and ζ are the 14-3-3 α and δ, respectively (66). Studies have also shown casein kinase 1 to phosphorylate 14-3-3 ζ and τ isoforms (76). However, the role of phosphorylation in the physiological regulation of 14-3-3 function is not yet clear.

2.3.5.3 Regulation by Subcellular Localization

Localization of 14-3-3 to cytoplasm, nucleus, various membranes, and cytoskeletal and centrosome structures has been described (104). As for understanding the significance of differential localization for 14-3-3 function, only the cytoplasmic/nuclear partition has been determined (105). Further studies by Muslin and Xing (2000) have reported that 14-3-3 proteins promote the cytoplasmic localization of various binding partner proteins (106). The pathophysiological importance of changes in 14-3-3 expression and localization in conditions such as cancer and neurodegenerative diseases is unresolved (107).

2.3.5.4 Isoform-Specific Regulation

Although the residues lining the phosphopeptide-binding groove of the various 14-3-3 isoforms are markedly conserved, differences in the abilities of the individual isoforms to bind peptides and proteins have been described (75, 92, 106, 108, 109). The interaction of 14-3-3 with ligands can be affected by intracellular variation in 14-3-3 levels. It appears that specific isoforms are limiting in cells despite the relative abundance of 14-3-3. For example, overexpression of 14-3-3 isoforms enhances Raf-1 activity in HeLa and COS cells (110, 111) and inhibits PKC activity in Jurkat T cells (112, 113).

Multiple isoforms of 14-3-3 may serve to regulate the total 14-3-3 pool via unique transcriptional controls for each isoform. Modulation of individual 14-3-3 expression can serve as an effective mechanism for regulating 14-3-3 functions. In vascular smooth muscle cells, 14-3-3 γ is induced by serum and platelet-derived growth factor (114), whereas 14-3-3 ε is down-regulated during differentiation of mesenchymal cells (115). In human colorectal carcinoma cells, 14-3-3 σ overexpression is induced by DNA damaging agents in a p53-dependent manner, leading to G2 arrest, whereas 14-3-3 β overexpression is not (116). Moreover, the dynamic expression patterns of various 14-3-3 isoforms during mouse embryogenesis and neuronal development highlights the importance of each 14-3-3 in mediating cellular processes (115, 117).

2.3.6 Stratifin/14-3-3 σ

14-3-3 σ, also known as stratifin, is unique among the 14-3-3 family members in that it is expressed primarily in epithelial cells (118) and appears to have isoform-specific functions (119). 14-3-3 σ expression is coordinately up-regulated by both BRCA1, a tumour suppressor for breast and ovarian cancers, and p53 and contributes to the DNA-damage cell-cycle checkpoint mediated by these proteins (116, 119-121). Unlike other 14-3-3 family members, which inhibit cell-cycle progression by interacting with the Cdc25 phosphatases, 14-3-3 σ can induce cell cycle arrest at G2 by a mechanism that involves 14-3-3 σ binding to and sequestering cyclin-dependent kinase-1/cyclin B1 complexes in the cytoplasm (119, 122, 123). Recent studies show that 14-3-3 σ, while inducing cell cycle arrest, may also play a role in delaying the alternative pathway to apoptosis by affecting localization of the pro-apoptotic protein Bax (124) and interacting with telomerase (125).

14-3-3 σ protein levels are significantly reduced or negligible in various transformed cell lines and primary tumors of epithelial origin, including breast and gastric cancers and hepatocellular carcinomas (126-131). Studies have shown that the loss of 14-3-3 σ expression is due to methylation of its promoter (126, 132-134). Dellambra *et al* (2000) demonstrated that down-regulation of 14-3-3 σ contributes to the immortalization of primary epithelial cells (135). 14-3-3 σ or stratifin thus acts as a tumor suppressor and loss of its function may be a critical event in the progression of certain human cancers.

Thus far, all of the biological activities of stratifin have been identified as intracellular interactions and functions; however, Ghahary *et al* (2004) have demonstrated a novel extracellular form of stratifin. A keratinocyte-derived extracellular form of stratifin was isolated from keratinocyte-conditioned medium (KCM) and this extracellular form of stratifin was shown to potently stimulate MMP-1 in dermal fibroblasts (136). Stratifin cDNA was cloned into an expression vector and recombinant stratifin was generated to confirm the results. This finding highlights the importance of mesenchymal-epithelial communication in the physiological and pathological process of dermal healing.

2.4 Mitogen-Activated Protein Kinase (MAPK) Pathways

2.4.1 Introduction

Protein kinases are enzymes that covalently attach phosphate to the side chain of either serines, threonines, or tyrosines of specific proteins inside multicellular organisms. The effect of phosphorylating such proteins can ultimately result in changes of enzymatic activity, interaction with other proteins and molecules, location within the cell, and susceptibility to protease degradation (137). Mitogen-activated protein kinases (MAPKs) are a highly conserved family of enzymes involved in the transduction of extracellular signals into cellular response (138, 139). MAPKs form a highly integrated network required to achieve specialized cell functions controlling gene expression, metabolism, cell differentiation, cell proliferation, and cell death (140). These cytoplasmic enzymes are able to modulate the activities of target protein substrates by phosphorylating specific serine and threonine residues. Substrates include other protein kinases, phospholipases, transcription factors, and cytoskeletal proteins. There are three well characterized members of the human MAPK superfamily: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38.

The activation of MAP kinases is unique in that they require phosphorylation on both adjacent threonine and tyrosine residues that are separated by a single amino acid, a tripeptide motif (Thr-Xxx-Tyr) (141). This dual phosphorylation is achieved by the activity of specific kinases which are known as MAP/ERK kinases (MEKs) or

MAP kinase kinases. The specificity of MEK for MAP kinase is dependent on the certain amino acid that is present in the middle of the tripeptide motif; for ERK is glutamate (Thr-Glu-Tyr), for the JNK family is proline (Thr-Pro-Tyr), and for p38 is glycine (Thr-Gly-Tyr) (142-144). The activity of MEKs is in turn controlled by phosphorylation of MAP kinase kinase kinases (MAPKKK, MKKK, or MEKK), a family of serine/threonine kinases in which the c-Raf protooncogene is the most prominent member (Figure 2.6.3). The regulation of this family of enzymes and their substrate specificity is still only partially understood (145).

Once MAP kinases are activated, transcription factors in the nucleus or cytoplasm are phosphorylated and activated, leading to expression of certain target genes resulting in a biological response. Cross-talk between the various MAP kinase cascades consists of multiple interactions which serve to integrate responses and to moderate biological outputs. Studies have shown that MAP kinases have overlapping substrate specificities (146). Moreover, the phosphorylation of regulatory sites can be shared among multiple protein kinases (147, 148).

2.4.2 Extracellular Signal-Regulated Kinase (ERK)

Among the three MAPK cascades, ERK was the first identified and hence the best characterized pathway. ERK is an essential mediator of many vital biological responses including cell growth, cell proliferation, and survival. There are two ERK isoforms, ERK1 and ERK2, and they are also referred to as p44 and p42, respectively (149, 150). There are various stimuli which activate the ERK1/2 pathways, including growth factors, cytokines, viral infection, ligands for heterotrimeric guanine nucleotide-binding protein (G-protein)-coupled receptors, transforming agents, and carcinogens (137). This cascade follows the classical three-level MAPK sequential kinase activation process in which MEK1 and MEK2 function as upstream MAPKK and the Raf proteins as MAPKKK (151, 152). The Raf family is a highly conserved serine-threonine kinase that is activated through its interactions with Ras (153, 154). The G-protein Ras is activated through its interaction with Grb2-Sos (son of sevenless) complex, where Sos catalyzes the dislocation of guanosine diphosphate (GDP) with the subsequent formation of Ras-guanosine triphosphate (GTP) complex (155). Only the GTP-bound form of Ras can bind to and activate Raf, thus recruiting Raf to the membrane where it undergoes phosphorylation (140, 156, 157). Mutations that convert Ras to an activated oncogene are common in many mammalian tumors. Oncogenic Ras continually activates the ERK1 and ERK2 signalling pathways, which ultimately contributes to increased proliferation observed in tumor cells. Therefore, inhibitors of the ERK pathway such as the compound, PD98059, which inhibits MEK and subsequent activation of ERK, are potential anticancer agents that are emerging into clinical trial studies (137).

Gene knockout experiments have demonstrated the importance of the ERK1/2 pathway (158). In mice, disruption of any of the three Raf proteins known to activate ERK1/2 consistently resulted in fatality. Furthermore, embryonic death was observed with signs of tissue necrosis when MEK1 was genetically targeted. A key role of ERK1/2 is the regulation of activating protein 1 (AP-1) family of transcription factors. Members of this family include c-Jun, c-Fos, and activating transcription factor 2 (ATF-2) which are phosphorylated and activated by ERK1/2. Recently, ERK activation has been shown to be critical for several inflammatory processes including T cell activation. Studies have shown that ERK-1 deficient mice exhibited defective thymocyte maturation and reduced expression of α and β chains of the T cell receptor mediated by the AP-1 family of transcription factors (145).

2.4.3 c-Jun N-terminal Kinase (JNK)

Originally isolated and identified as a 54 kDa stress-activated protein kinase (SAPK), JNK is a cell stress activated MAPK involved in regulation of cell proliferation and apoptosis. JNK is recognized as the MAPK by stress-induced stimuli such as lipopolysaccharide (LPS), interleukin (IL)-1, tumor necrosis factor (TNF), ultraviolet radiation, and osmotic stress (141). There are three genes that encode JNK protein kinases, JNK-1, JNK-2, and JNK-3 (159). While JNK3 appears to be expressed only in heart, brain, and testis, JNK1/2 are ubiquitously expressed (141).

The two MAPKK upstream activators of JNK are MKK7, which is primarily activated by cytokines, and MKK4, primarily activated by environmental stress (141, 160). The JNK pathway is activated by a large group of MAPKKKs, including those of the MEKK group (MEKK1-4) (161). The diversity of MAPKKKs allows a vast range of stimuli to activate this MAPK pathway. Further upstream of MAPKKK, the JNK pathway can be activated through Cdc42/Rac/PAK network of proteins. As members of the rho family of GTP-binding proteins, Cdc42 and Rac play essential roles in cytoskeletal rearrangement. Activated Cdc42 and Rac subsequently activates PAK, also known as p21-activated kinase, which subsequently activates the JNK cascade (162-164).

Activated JNK is able to phosphorylate the transcription factor, Elk-1, in addition to two AP-1 transcription components, c-Jun and ATF-2. Gene knockout studies of JNK1-3 in mice show defects in immune responses and apoptosis, while its isolated embryonic fibroblasts demonstrate defective AP-1 transcription activity, decreased proliferation, and resistance to stress induced apoptosis (165). Other genetic modification studies show that JNK plays an important role in TNF expression (166-169) as well as T-cell proliferation, differentiation, and IL-2 production (170).

2.4.4 p38 MAPK

The p38 MAPK pathway is associated with many physiological activities including cell growth, cell differentiation, cell death, and the control of inflammatory response. p38 is activated by hormones, ligands for G-protein-coupled receptors as well as LPS, ultraviolet exposure, physiological and osmotic stress (171-173). In immune cells, p38 MAPK is activated by a variety of cytokines (IL-1, IL-2, IL-7, IL-17, IL-18, TGF-β, and TNF) (174). There are five isoforms of p38 kinases: α, β, β2, γ, and δ (143, 175-178). Expression of each isoform differs in different tissues. While p38α and p38β isoforms are ubiquitously expressed (174), p38γ is expressed predominantly in skeletal muscle and p38δ in lungs, kidneys, pancreas, small intestine and testis (179). In addition, p38α is the major isoform activated in most inflammatory cells as it is expressed in high levels in leukocytes and bone marrow (179).

The upstream MAPK kinases responsible for p38 activation are MKK3, MKK4, and MKK6. As it is with the expression of the p38 MAPK isoforms, the preferred upstream kinase is different for each isoform and cell type (161). In leukocytes, MKK3 is abundantly expressed whereas MKK6 is not (180-183). Moreover, LPS selectively activates p38a via MKK3 and not MKK4 nor MKK6 (177). While MKK3 activates only p38α/γ/δ, MKK6 is a common activator of p38α/β/β2/γ/δ. Further upstream of the p38 pathway, there are multiple serine/threonine kinases that act as MAPKKK which explains why this cascade can be activated by a wide variety of stimuli. Two of the MAPKKKs that are responsive to the receptor-mediated environmental stimuli of the p38 pathway are TAK1 and ASK-1 (140).

The downstream substrates for p38 MAPK are transcription factors such as ATF-2, Sap-1, and myocyte-enhancer factor 2 (MEF2) (184, 185). ATF-2 binds to cyclic AMP responsive elements (CREs) and subsequently forms heterodimers with c-Jun to regulate the expression of various inflammatory proteins (186, 187). p38 MAP kinase can also directly phosphorylate kinases, such as MAP kinase-activated protein kinase-2 (MAPKAP-K2) and MAPKAP-K3 (188-192). MAPKAP-K2/3 are both serine protein kinases that activate cyclic AMP responsive elements binding protein (CREB) and heat shock protein-27 (HSP-27) (192-194). As an actin binding protein, HSP-27 plays an important role in the repair of actin cytoskeleton, inhibition of apoptosis, and anti-inflammatory activity (192, 195, 196). Other substrates for p38 phosphorylation are cytosolic phospholipases A2, MAP kinase-interacting kinase-1 (MNK-1), Elk-1, CHOP, and myelin basic protein (147, 148, 188, 189, 197-200).

2.4.5 Crosstalk between MAP Kinase Pathways

Crosstalk between the different MAP kinase pathways exists as the intracellular protein kinase pathways are part of a large interactive network. MAPK cascades share many upstream as well as downstream kinases in addition to transcription factors, all of which interact and integrate these pathways (Figure 2.6.4). An example of this interconnected system is in the MAPK regulation of the AP-1 transcription factor (201). AP-1 activity that is regulated by MAPK involves increasing transcription of both c-Fos and c-Jun (202). ERK, JNK, and p38 have the ability to phosphorylate and activate the ternary complex factor Elk-1, which acts to induce c-Fos transcription. Furthermore, both ERK and p38 can activate Sap-1, another ternary complex factor involved in the stimulation of c-Fos transcription (185). JNK can phosphorylate and increase transcription activity of c-Jun (141), while p38 can phosphorylate ATF-2 and CREB (via MAPKAP-K2/3) and thereby modulate AP-1 activity (186, 187, 203).

Zhang *et al* demonstrated that p38 is directly involved in the stress-induced inhibition of ERK1 and ERK2 (204). Furthermore, the JNK and p38 pathways may converge through MKK4 activation (160, 205). Activation of one MAP kinase can cause activation of dual specificity phosphatase, which not only dephosphorylates and deactivates activated MAP kinase but also other MAP kinases. Therefore, crosstalk can result in cooperative interaction as is the case with one activated MAP kinase leading to the activation of another isoform. In contrast, complex interaction of the various pathways can result in lateral inhibition of MAP kinase by different isoforms as well.

2.5 Thesis Aims and Experimental Rationale

One of the main questions in wound healing research is why and how any delay in epithelialization increases the frequency for developing fibrotic conditions developed following deep trauma, surgical incision, electrical and thermal injury. Recently, our laboratory has discovered a keratinocyte releasable factor which may function as a termination signal for the dynamic process of wound healing. When dermal fibroblasts are treated with this factor, a dramatic increase in MMP-1 production is observed, which may play a major role in remodelling of the wound site. Thus, the level of expression of this factor could potentially determine the quality of the end-point of the healing process. Subsequently, this protein was isolated and identified to be 14-3-3 σ, which is also known as stratifin. The gene of this protein was then cloned and its corresponding protein was expressed in *E. coli*.

The stimulatory effect of stratifin on MMP-1 expression has so far been validated in a fibroblast culture system. This finding indicates that keratinocyte-fibroblast cross communication may be critical for regulation of the wound healing process. Thus, the lack of extracellular stratifin at the wound site, due to any delay in wound epithelialization such as severity of injury or infection, could potentially lead to the development of dermal fibrosis and hypertrophic scarring. Although the MMP-1 stimulatory effect of stratifin has been validated, the question of its molecular mechanism by which it signals MMP-1 induction and also the regulation of its efficacy in dermal fibroblasts have yet to be determined.

In this study, I hypothesize that stratifin-induced MMP-1 expression is mediated by a MAPK kinase pathway and this induction of MMP-1 is antagonized by a regulatory hormone.

Therefore, the specific aims of the study were to:

I. identify any factors which may regulate stratifin as well as its efficacy on expression of collagenase in dermal fibroblasts.
II. identify signaling pathway elements mediating stratifin stimulation of fibroblast MMP-1 expression by examining the three distinct MAPK pathways: ERK1/2, JNK, and p38 as well as the expression of the main components of the AP-1 dimers, c-Jun and c-Fos, which is mediated by distinct MAPK pathways.
III. identify other functional groups of the signalling pathway involved in the up-regulation of collagenase in dermal fibroblasts by stratifin with the use of a human MAP Kinase signalling pathway gene array assay.

2.6 Figures

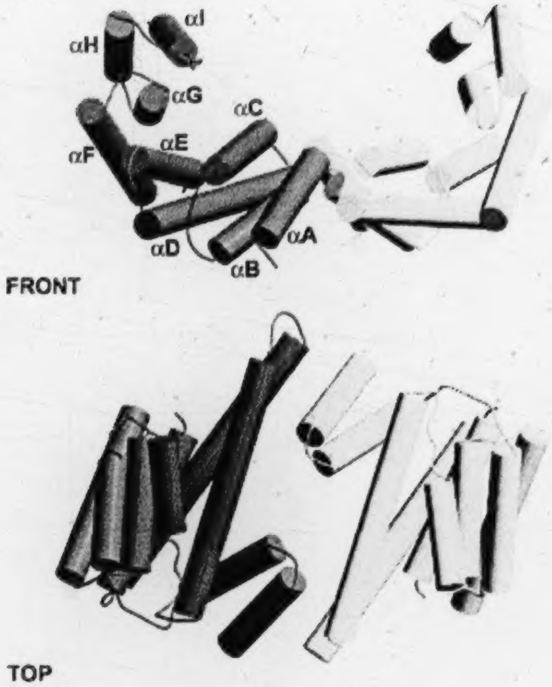
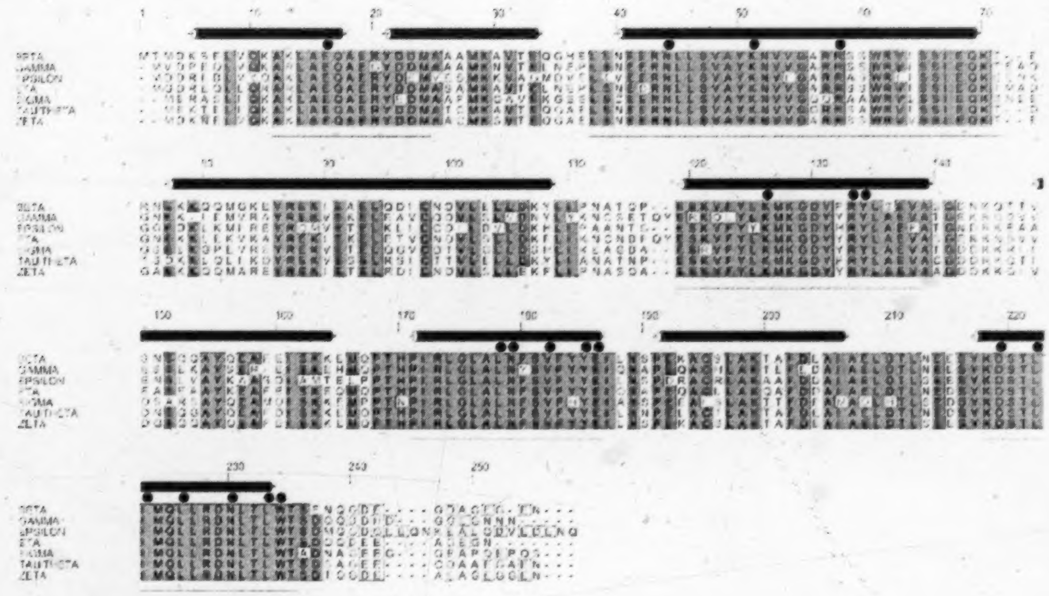

αI
αH
αG
αC
αF
αE
αA
αD
αB
FRONT
TOP

Figure 2.6.1 The structure of 14-3-3 in two orthogonal views. Helices αA–αI are shown as cylinders (99).


BETA
GAMMA
EPSILON
ETA
SIGMA
TAU/THETA
ZETA
1
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
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240
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Figure 2.6.2 Sequence alignment of human 14-3-3 isotypes. Residues conserved in at least six of the seven isotypes are shaded gray. The structure of 14-3-3 is indicated by helices above the alignment. Five conserved sequence blocks within the 14-3-3 family are indicated by a thin line below the alignment. Residues within the binding cleft that interact with peptide ligands are indicated by filled circles. Acidic residues within the divergent C-termini are boxed. (99).

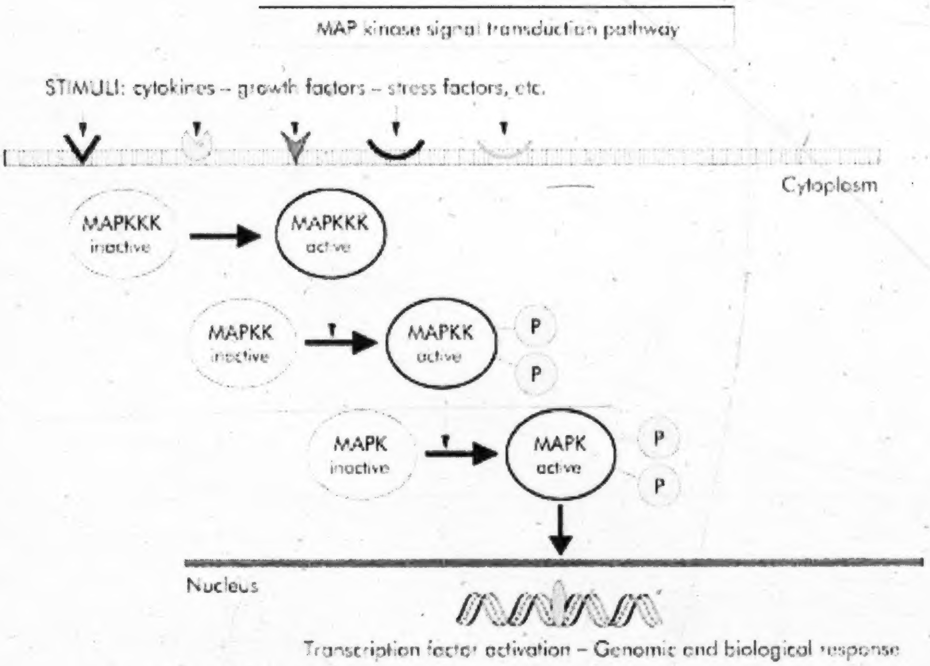
MAP kinase signal transduction pathway
STIMULI: cytokines – growth factors – stress factors, etc.
Cytoplasm
MAPKKK inactive
MAPKKK active
MAPKK inactive
MAPKK active
P
P
MAPK inactive
MAPK active
P
P
Nucleus
Transcription factor activation – Genomic and biological response

Figure 2.6.3 MAP kinase signal transduction model. Various extracellular stimuli are able to activate mitogen activated protein (MAP) kinases after receptor-ligand interactions. MAPK, MAP kinase; MAPKK, MAP kinase kinase; MAPKKK, MAP kinase kinase kinase. (145)

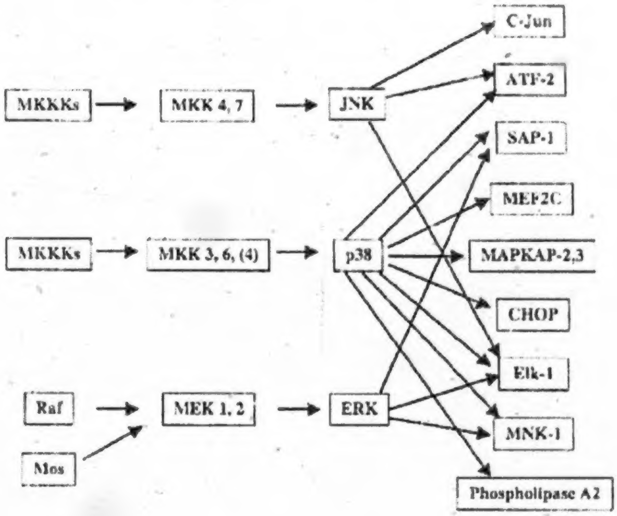
MKKKs
MKK 4, 7
JNK
MKKKs
MKK 3, 6, (4)
p38
Raf
Mos
MEK 1, 2
ERK
C-Jun
ATF-2
SAP-1
MEF2C
MAPKAP-2,3
CHOP
Elk-1
MNK-1
Phospholipase A2

Figure 2.6.4 Crosstalk between mitogen activated protein (MAP) kinase pathways in downstream substrates. MKKK, MAPK kinase kinase; MKK, MAPK kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase. (206)

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CHAPTER 3:

INSULIN SUPPRESSES COLLAGENASE STIMULATORY EFFECT OF STRATIFIN IN DERMAL FIBROBLASTS

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3.1 ABSTRACT

A delicate balance between synthesis and degradation of extracellular matrix (ECM) by matrix metalloproteinases (MMPs) is an essential feature of tissue remodeling. Ghahary *et al* (2004) have recently demonstrated that keratinocyte releasable stratifin, also known as 14-3-3 σ protein, plays a critical role in modulating MMP-1 mRNA expression in human dermal fibroblasts. In this study, I further characterized the MMP-1 stimulatory effect of stratifin in dermal fibroblasts and evaluated its effect in the presence and absence of insulin. My data indicate that stratifin increases the expression of MMP-1 mRNA more than 20 fold in dermal fibroblasts grown in either Dulbecco's modified Eagle's medium (DMEM) plus 2% or 10% fetal bovine serum (FBS). MMP-1 stimulatory effect of stratifin was completely blocked when fibroblasts were cultured in test medium consisting of 50% keratinocyte serum-free medium (KSFM) and 50% DMEM. The MMP-1 antagonistic effect of test medium was directly proportional to the volume of KSFM used. As this medium contained insulin, I then evaluated the MMP-1 stimulatory effect of stratifin in dermal fibroblasts in the presence and absence of insulin. The results revealed that stratifin significantly increased the expression of MMP-1 mRNA/18S (*$p<0.05$, $n=3$) ratio, while insulin significantly decreased the expression of MMP-1 mRNA/18S (*$p<0.05$, $n=3$) ratio. The insulin antagonistic effect on MMP-1 mRNA expression was time and dose dependent. The maximal antagonistic effect of insulin was seen at 36 hr post treatment. In conclusion, stratifin stimulates the expression of MMP-1 mRNA expression in dermal fibroblasts and this effect is suppressed by insulin treatment.

3.2 INTRODUCTION

The process of wound healing as well as the maintenance of structural integrity of normal skin require a delicate balance between synthesis and degradation of extracellular matrix (ECM). Proteolytic breakdown of ECM by a large family of enzymes known as matrix metalloproteinases (MMPs) is an essential feature of tissue remodeling in several physiological situations, such as developmental tissue morphogensis, angiogenesis and tissue repair. Any aberrations in expression or degradation of ECM components may result in either retarded wound healing, as is seen in diabetic and the elderly population, or over-healing wounds such as fibroproliferative disorders frequently seen in surgical incision, traumatic wounds and severe thermal injury. Since MMPs are involved in ECM turnover and connective tissue remodeling, an imbalance in expression of MMPs has been implicated in a number of pathological conditions such as dermal fibrosis (1), rheumatoid arthritis, atherosclerosis, pulmonary emphysema and tumor invasion and metasis (2, 3).

The MMP family consists of 25 zinc-dependent and calcium-dependent proteinases in mammalian system. According to their substrate specificity, primary structure and cellular localization, MMPs are subdivided into five different classes: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs (4). The basal expression of most MMPs in cultured cells is low, and these enzymes are transcriptionally induced by a variety of growth factors and cytokines (5).

Chronic non-healing wounds are common causes of morbidity among diabetic patients. Impaired wound healing, such as in foot ulcers, is indeed correlated with the diabetic state (6-9). Diabetic rats exhibit adequate wound healing when their blood glucose levels have been normalized using insulin (10). Several of the complications associated with both insulin-dependent type I diabetes mellitus and non-insulin-dependent type II diabetes mellitus are characterized, in part, by alterations in the amount and composition of ECM proteins (11). Impaired healing is also caused by alterations in growth factors and protease expression (12-14). Chronic wounds have been shown to possess increased collagenolytic levels and activity (15). Moreover, *in situ* hybridization of chronic wound tissues has revealed elevated MMP-1 transcripts in fibroblasts underlying the non-healing epithelium (16). The protein levels of MMP-1 are three-fold higher in chronic wound fluids than is observed for peak levels of fluids from healing dermal wounds (17). Impaired wound healing, therefore, exhibits excessive tissue destruction mediated by proteases. Several pathogenic mechanisms have been suggested to be involved in the development of diabetic skin complications; however, the exact molecular mechanism through which the level of MMP-1 remains high is not known.

Insulin has been shown to regulate the expression of specific genes by affecting transcription, mRNA stability or mRNA translation (18). The genes regulated by insulin encode proteins involved in a vast array of biological phenomena not only observed in the tissues associated with the metabolic actions of insulin, namely liver, muscle and adipose tissue, but also in tissues not commonly associated with these metabolic effects (19). The effect of insulin on MMP-1 expression in HeLa cells (20), Chinese hamster ovary (21), and NIH 3T3 (22) have been studied.

Ghahary *et al* (2004) have recently identified stratifin, or 14-3-3 σ, as a potent MMP-1 stimulatory factor in dermal fibroblasts (23). In this study, I propose that low levels of insulin may be one of the contributing factors of elevated levels of MMP-1. Therefore, I hypothesize that insulin may be an antagonist of stratifin and its MMP-1 stimulatory effect in fibroblast. Here, I further confirmed the MMP-1 stimulatory effect of stratifin in dermal fibroblasts grown under different experimental conditions. My data also revealed that insulin suppresses the MMP-1 mRNA expression in dermal fibroblasts in the absence and even presence of a potent MMP-1 stimulatory factor, 14-3-3 σ.

3.3 MATERIALS AND METHODS

Fibroblast cell culture:

Cultures of human dermal fibroblasts were established as described previously (24). In brief, punch biopsy samples were prepared from human adult dermal fibroblasts. The tissue was collected in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island , New York) plus 10% FBS supplemented with antibiotic-antimycotic preparation (100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) (Gibco). Specimens were dissected and minced into small pieces less than 0.5 mm in diameter, washed six times with DMEM, and distributed into 60 X 15-mm culture dishes (Corning Inc., Corning, NY), four pieces per dish. After 4 weeks of incubation, the cells were released from dishes by brief treatment with 0.1% trypsin (Life technologies Inc., Gaithersburg, MD) and 0.02%

ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) in PBS (pH 7.4) and reseeded into 75-cm^2 flasks (Corning Inc). Upon reaching confluence, the cells were subcultured at a ratio of 1:6 by trypsinization. Fibroblasts from passages 3-8 were used for this study.

Preparation of human recombinant stratifin (14-3-3 σ):

Procedure of human recombinant 14-3-3 σ protein preparation were established as described previously (23). Briefly, the cDNA of 14-3-3 σ from human keratinocytes was cloned into pGEX-6P-1 expression vector (Amersham/Pharmacia Biotech) and transformed into protein expressing bacteria, BL-21 (DE3) (Novagene). A single positive clone was grown in 100 ml of LB medium containing 50 μg/ml of ampicillin for 4-6 hours at 29 °C until an OD_{600nm} of 0.4-0.6 was reached. Bacteria were then diluted to 1:10 with LB medium plus 0.1 mM IPTG for 12 hrs. To purify the protein, bacteria were centrifuged and lysed with 50mM Tris-HCl (pH 7.4) containing 10mM EDTA, 5mM EGTA, protease inhibitor cocktail (Sigma), 1% Triton X-100, and 0.5% IGEPAL CA630. Cell lysate was passed through a Glutathione Sepharose 4B affinity column and subsequently washed with PBS containing 0.1% Triton X-100 until an OD_{280nm} reached zero. GST-fused 14-3-3 σ was digested using PreScission protease according to manufacturer's procedure (Amersham/Pharmacia Biotech). GST-free 14-3-3 σ was then eluted, dialysed against PBS and then concentrated with Centricon (Millipore). The sequence of protein was validated on a Bruker REFLEX III mass spectrometer (Bremen/Leipzig, Germany) using MALDI in positive ion mode.

Treatment of dermal fibroblasts:

For each experiment, fibroblasts were seeded into 60 X 15-mm petri dishes (Corning Inc.). Confluent fibroblasts were then rinsed with phosphate-buffered saline (PBS) before various growth media were added. To evaluate the efficacy of stratifin in different growth media, cells were incubated in either our test medium, consisting of 50% keratinocyte serum-free medium (KSFM) (Gibco) and 50% DMEM without FBS, DMEM plus 2% FBS or DMEM plus 10% FBS in the presence and absence of stratifin (2.5 μg/ml). Based on previous experiments, the stratifin protein concentration of 2.5 μg/ml was the optimal dose for the stimulation of MMP-1 mRNA expression in dermal fibroblasts for 24 hr incubation period. Various ratios of DMEM/KSFM plus 2% FBS were also used to test the effect of KSFM on stratifin efficacy on MMP-1 mRNA expression. To determine the optimal effective dose of insulin on the expression of MMP-1 mRNA expression, various concentrations (0, 0.001, 0.01, 0.1, 1, 10 mU/ml) of human insulin, in the presence or absence of stratifin (2.5 μg/ml), were added and cells were incubated in DMEM plus 2 % FBS for 48 hrs. Human biosynthetic insulin was purchased from Novo Nordisk Canada Inc. (Mississauga, ON). In subsequent experiments, the optimum dose of insulin determined previously by the dose-response experiment (10 mU/ml) was added and cells were incubated for 48 hrs. Another set of cell cultures with no stratifin treatment served as controls. To determine the time course of the treatments of stratifin, insulin, and a combination of both on MMP-1 mRNA expression, the cells were then incubated with 2.5 μg/ml of stratifin, 10 mU/ml of insulin or both for the indicated times.

RNA isolation and Northern blot analysis:

Fibroblasts were harvested with 400 μl of 4 M guanidium isothiocyanate (GITC) solution and total RNA from each group was isolated by the guanidium isothiocyanate/ CsCl procedure of Chomczynski and Sacchi (1987) using phenol:chloroform (1:1). Total RNA from each individual fibroblast culture was then separated by electrophoresis (10μg per lane) on a 1% agarose gel containing 2.2 M formaldehyde and was blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California). The blots were baked for 2 hr at 80°C under vacuum and prehybridized for 4 hr at 45 °C in a prehybridization solution. Hybridization was performed at 45 °C in the same solution, using collagenase and 18S ribosomal RNA cDNA probes. The cDNA probes were labeled with P-α^{32}-dCTP by nick translation. The filters were washed initially at room temperature with 2X sodium citrate/sodium chloride buffers and 0.1% sodium dodecylsulfate for 1 hr and finally washed for 20 min at 65 °C in 0.1X sodium citrate/sodium chloride buffer and 0.1% sodium dodecylsulfate. Autoradiography was performed by exposing Kodak X-Omat film to nitrocellulose filters at -80 °C in the presence of an intensifying screen. Quantitative analysis of autoradiographs was accomplished by densitometry. The cDNA probe for MMP-1 and 18S ribosomal RNA were obtained from the American Type Culture Collection (Rockville, MD). Each experiment was performed at least twice to ensure reproducibility of the results.

Statistical analysis

Autoradiograms of northern analysis were quantified by densitometry and data expressed as MMP-1 mRNA/18S rRNA. The statistical significance of differences in MMP-1 mRNA expression between treated and untreated dermal fibroblasts was tested with Student's unpaired two-tailed *t*-test; p values < 0.05 and < 0.01 were considered significant and very significant, respectively.

3.4 RESULTS

Efficacy of stratifin on MMP-1 mRNA expression in fibroblast

To analyze the efficacy of stratifin in various growth media, dermal fibroblasts were incubated in DMEM with either 2% or 10% of FBS as well as 50/50 medium consisting of 50% DMEM and 50% KSFM with no additives. The results showed that stratifin increases the expression of MMP-1 mRNA more than 20 fold in fibroblasts grown in DMEM + FBS medium. However, this strong MMP-1 stimulatory effect of stratifin in dermal fibroblasts was blocked when they were cultured in 50/50 medium **(Fig 3.8.1*A*)**. Fibroblasts treated with stratifin in DMEM in the presence of either 2% or 10% FBS induced strong MMP-1 mRNA expression. Several experiments were then conducted to determine why MMP-1 stimulatory effect of stratifin is abrogated when cells are grown in 50/50 medium. As 50/50 medium contained a low concentration of calcium (0.96 mM) and had no FBS, we cultured fibroblasts in the presence and absence of FBS and added calcium to 50/50 medium at final concentration comparable to that of DMEM (1.92mM). The results of Northern blot analysis revealed that neither the presence of 2 % FBS nor increased calcium concentration in 50/50 medium restored the MMP-1 stimulating effect of stratifin on dermal fibroblasts **(Fig 3.8.1*B*)**.

KSFM inhibits the MMP-1 stimulatory effect of stratifin in fibroblasts

Since FBS and increased calcium concentration did not restore the efficacy of stratifin, we examined whether the inhibitory effect of KSFM is proportional to its volume used in 50/50. We, therefore, treated dermal fibroblasts with stratifin in various ratios of DMEM/KSFM media (50/50, 70/30, 90/10) plus 2% FBS and determined its effect on MMP-1 mRNA expression by Northern analysis. The results showed that as the ratio of DMEM/KSFM increased, the efficacy of stratifin was restored to its maximal MMP-1 stimulatory effect **(Fig 3.8.2)**. In 100% DMEM media plus 2% FBS, stratifin had a maximal MMP-1 stimulating effect in dermal fibroblasts. To show that the apparent alterations in the expression of MMP-1 mRNA in response to stratifin treatment in various DMEM/KSFM media were not due to variations in RNA loading, the same blots were re-hybridized with cDNA for 18S RNA. This result shows that KSFM reduces the potent MMP-1 stimulatory effect of stratifin on dermal fibroblasts.

Insulin reduces the efficacy of stratifin on mRNA expression of MMP-1

Our results indicate that there is an antagonist(s) in KSFM that reduces the efficacy of stratifin on MMP-1 mRNA expression in fibroblasts. Since insulin is a key ingredient in keratinocyte growth media, we examined whether insulin could be a potential inhibitory factor of stratifin efficacy. To determine the optimal effective dose of insulin for fibroblast treatment, fibroblasts were treated in DMEM plus 2% FBS with various concentrations (0, 0.001, 0.01, 0.1, 1, 10 mU/ml) of insulin in the presence and absence of 2.5 μg/ml of stratifin for 24 hours **(Fig 3.8.3*A*)**. The results showed an antagonistic effect of insulin on efficacy of stratifin to stimulate MMP-1 mRNA expression in dermal fibroblasts and this effect was dose dependent. To show equal RNA loading, we also hybridized the same blot with 18S cDNA to control for RNA loading. Densitometry results revealed that insulin reduces MMP-1 mRNA/18S ratio in both stratifin treated and non-treated fibroblasts **(Fig 3.8.3*B*)**. A dose of 10 mU/ml of insulin had a maximum inhibitory effect and it was therefore used for subsequent experiments.

For statistical analysis, different strains of human dermal fibroblasts cultured from various individuals were either untreated (control) or treated for 48 hours with 2.5 μg/ml of stratifin, 10 mU/ml of insulin or a combination of both. Densitometry results from the three separate experiments shown in **Fig 3.8.4*A*** indicate that stratifin significantly increases, while insulin significantly decreases, the expression of MMP-1 mRNA/18S. (*$p<0.05$, n=3) **(Fig 3.8.4*B*)**. Statistical evaluation between stratifin and insulin treated samples as well as between insulin and both treatment samples showed significant differences in expression of MMP-1 mRNA/18S (**$p<0.01$, n=3; ***$p<0.05$, n=3, respectively). In addition, MMP-1 mRNA level of stratifin treated samples was markedly reduced in insulin treated cells.

In the same experimental setting, 10 mU/ml of insulin was used to determine the time required for the onset of insulin effects on MMP-1 mRNA expression. Fibroblasts were treated with either stratifin alone, insulin alone, or a combination of both for the indicated time intervals. Northern analysis of MMP-1 mRNA and 18S rRNA expression was performed **(Fig 3.8.5*A*)** and the results revealed an increase in MMP-1 mRNA expression by stratifin treatment with its peak intensity at 24 hours **(Fig 3.8.5*B*)**. In contrast, treatment of insulin reduced collagenase mRNA expression with maximal effect at 36 hours. A combination of both reduces the expression MMP-1 mRNA below its normal level. This finding reveals that insulin markedly reduces the efficacy of stratifin in stimulation of MMP-1 expression in a time dependent fashion in fibroblasts.

3.5 DISCUSSION

The 14-3-3 σ proteins are a ubiquitous family of acidic, eukaryotic class of highly conserved molecular chaperones in the 25-30 kDa range of molecular weight. There are seven known mammalian isoforms, α/β, γ, ε, π, σ, τ(θ) and ζ (25). Since the discovery of the first 14-3-3 protein in 1967 (26), the members of the 14-3-3 protein family have been re-discovered repeatedly based on their new biological activities, primarily in signal transduction pathways. They have been identified as activators of tryptophan and tyrosine hydroxylase (27, 28) and PKC inhibitors (29). Subsequent studies identified the 14-3-3 proteins as molecules that interact with PKCs, Raf family members and now more than 100 other intracellular proteins with critical biological functions (30, 31) including cellular response to DNA damage and cell cycle regulation (32-34).

Thus far, studies on 14-3-3 proteins have characterized its function as intracellular protein. An extracellular form of 14-3-3 protein was reported to be present in cerebrospinal fluid (CSF) and associated with prion diseases such as Creutzfeldt-Jakob disease and other neurological disorders (35, 36). However, biological function of extracellular 14-3-3 proteins has not been explored until recently by our research group. In a previous study performed by Ghahary *et al* (2004), a keratinocyte-derived MMP-1 stimulating factor for dermal fibroblasts was identified as an extracellular form of stratifin, also known as 14-3-3 σ (37). Since little is known about extracellular effects of stratifin, this study was conducted to further confirm the MMP-1 stimulating effects of recombinant form of stratifin in fibroblasts grown under different culture conditions and to evaluate its efficacy by a MMP-1 modulating factor such as insulin. My findings revealed that stratifin increases the expression of MMP-1 and this effect is blocked by growing fibroblasts in our test medium called 50/50. My observations indicated this inhibitory effect was due to insulin, one of the major ingredients of this medium. An addition of insulin to stratifin containing medium reduces the MMP-1 stimulatory effect of stratifin in fibroblasts.

While insulin has long been known to regulate intracellular metabolism by altering the activity or intracellular location of various enzymes, the regulation of gene expression by insulin has been recognized in recent studies (18). The effect of insulin on HeLa cells (20), Chinese hamster ovary (21), and NIH 3T3 (22) have been shown to stimulate MMP-1 expression via the AP-1 motif. However, in this study, my results have revealed that insulin suppresses the transcription of MMP-1 in dermal fibroblasts in the presence or absence of stratifin. This contrasting result from previous studies may be due to the use of different cell types other than skin fibroblasts. Insulin seems to have differential roles in different cell types. For example, insulin has previously been shown to stimulate albumin mRNA expression in diabetic rats (38, 39) as well as in short term primary hepatocyte cultures (40). However, the effect of insulin on albumin gene transcription is reversed in cultured H4-II-E rat hepatoma cells, showing a decrease in the levels of albumin mRNA (41). Thus, insulin has differential effects depending on its target cells.

There are at least eight distinct consensus insulin response sequence (IRSs) identified through which insulin can regulate gene transcription. While most IRSs including the activator protein 1 (AP-1) motif, the serum response element, the Ets motif, and the E-box motif have been shown to mediate stimulatory effects of insulin on gene transcription, the phosphoenolpyruvate carboxykinase (PEPCK)-like motif mediates a contrasting effect of insulin on transcription of genes. The PEPCK motif, an element with the consensus sequence T(G/A)TTT(T/G)(G/T), mediates the inhibitory effect of insulin on transcription of genes that encode PEPCK, insulin-like-growth-factor-binding protein 1 (IGFBP-1), tyrosine aminotransferase and the glucose-6-phosphatase (G6Pase) catalytic subunit (19).

Previous studies by Endo *et al* (2003) revealed that PDGF suppresses the expression of MMP-1 mRNA in dermal fibroblasts through activation of p38 MAPK (42). It is also reported that insulin induces p38 activity in cultured myoblasts which are important for cytoskeletal rearrangement and myoblast differentiation (43, 44). Consistent with these findings, Somwar *et al* (2001) and Sweeney *et al* (1999, 2001) showed that insulin stimulated p38 activity in L6 myotubes is essential for insulin-induced glucose transport (45-48). However, this may not be the case for other cell strains examined. For example, in fetal neurons, insulin inhibits the p38 pathway as well as apoptosis (49). Although little is know about the activation of any signal transduction pathway in response to either stratifin or insulin in skin cells, we anticipate that the p38 MAPK pathway may be involved in the modulation of MMP-1 expression by insulin and stratifin treatment in dermal fibroblasts. With the use of specific MAPK inhibitors, our preliminary results have shown stratifin-induced MMP-1 expression is mediated by p38 MAPK (data not shown). Therefore, insulin may antagonize the expression of MMP-1 in dermal fibroblasts by inhibition of p38 pathway. At present, this is under our current investigation.

In conclusion, the findings of this study further confirmed the MMP-1 stimulatory effect of recombinant stratifin in dermal fibroblasts. This effect was markedly antagonized by administration of insulin. Further studies may identify other factors which may also antagonize the stimulatory effect of stratifin on MMP-1 mRNA expression in dermal fibroblasts. The mechanism by which insulin antagonizes the MMP-1 stimulating effect of stratifin is also currently being explored in our laboratory.

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3.7 FIGURE LEGENDS

Figure 3.8.1. Inhibition of stratifin collagenase mRNA stimulatory effect on dermal fibroblast by 50/50 medium.

Fibroblasts were treated with or without 2.5µg/ml of stratifin in 50/50 (DMEM/KSFM), DMEM +2% FBS, or DMEM +10% FBS for a period of 48 hrs. The total RNA was extracted and Northern blot analysis was performed to determine the expression of collagenase mRNA. The same blots were re-hybridized with cDNA specific for 18S ribosomal RNA and were used as a RNA loading control. **Panel A,** depicts the inhibition of stratifin collagenase stimulatory effect on fibroblasts in 50/50 medium. **Panel B**, depicts the finding that neither the presence of FBS nor increased calcium concentration in 50/50 medium restore the efficacy of stratifin. Concentration of calcium was elevated to that of DMEM (1.92mM).

Figure 3.8.2. Inhibitory effect of KSFM on stratifin efficacy on collagenase mRNA expression in dermal fibroblast.

Fibroblasts were treated with or without 2.5µg/ml of stratifin in various ratios of DMEM and KSFM plus 2% FBS for a period of 48 hrs. The total RNA was extracted and Northern blot analysis was performed to determine the expression of collagenase mRNA. The same blots were re-hybridized with cDNA specific for 18S ribosomal RNA and were used as a RNA loading control. This figure depicts the inhibition of stratifin collagenase stimulatory effect on fibroblasts by increasing amounts of KSFM.

Figure 3.8.3. Dose-dependent inhibitory effect of insulin on stratifin efficacy on collagenase mRNA expression in dermal fibroblasts

Fibroblasts were treated in DMEM plus 2% FBS with 0, 0.001, 0.01, 0.1, 1, 10 mU/ml of insulin for a period of 48 hrs. Cells were harvested and total RNA extracted prior to Northern blot analysis of collagenase mRNA expression. 18S ribosomal RNA was used as a loading control. **Panel A** depicts the pattern of collagenase mRNA and 18S rRNA expression in fibroblasts treated with 2.5μg/ml of stratifin plus various concentrations of insulin. Another set of fibroblasts with no stratifin treatment served as controls. **Panel B**, depicts densitometry data on the ratio of collagenase mRNA/18S as a function of various amounts of insulin treatments in the presence and absence of stratifin. Solid and open bars represent stratifin treated and non-treated (control) samples, respectively.

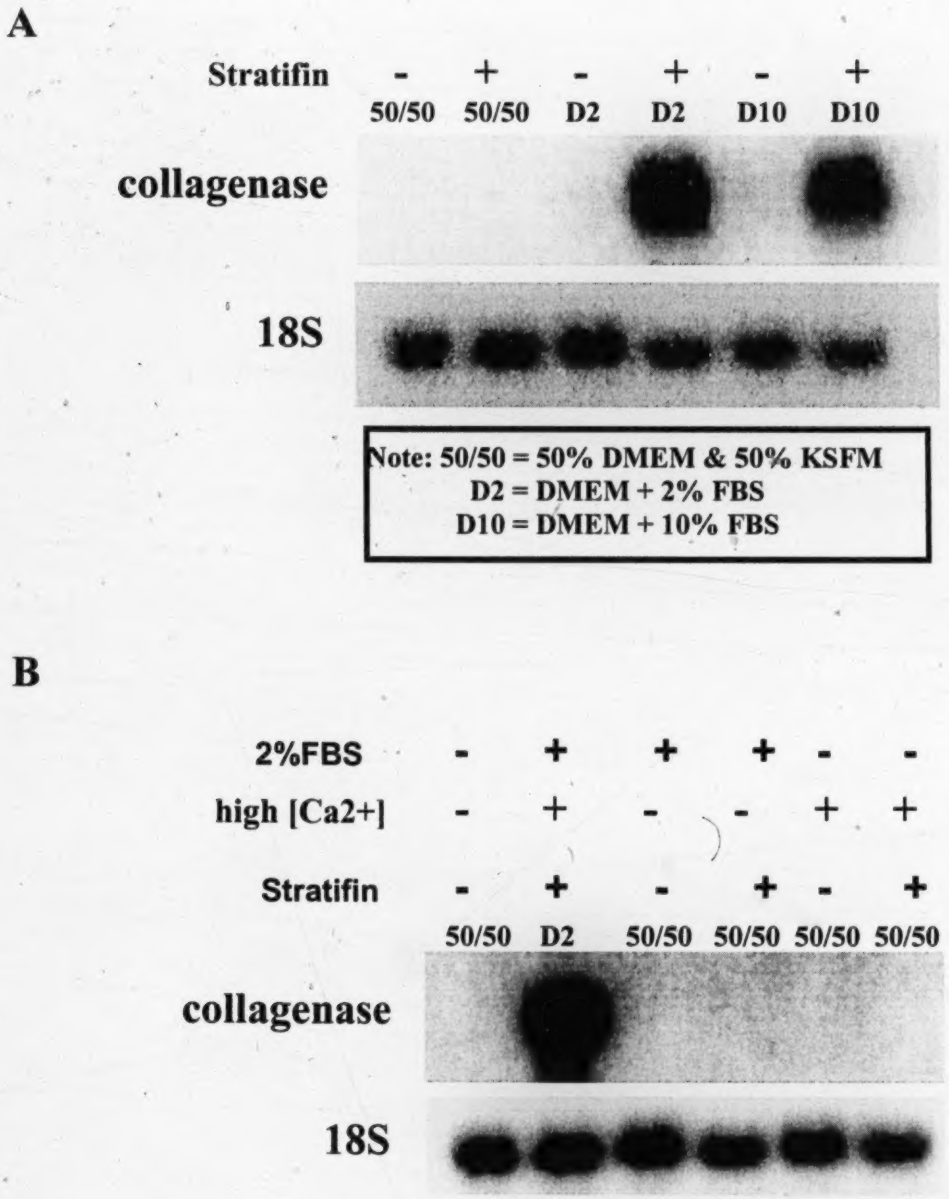
Figure 3.8.4. Insulin suppression of collagenase mRNA expression is responsive in different fibroblasts strains with and without stratifin treatment.

Three different strains of human fibroblasts in DMEM plus 2% FBS were untreated (N), treated with 2.5 μg/ml of stratifin (S), treated with 10 mU/ml of insulin (I), or both (S+I) for a period of 48 hrs. Northern analysis was performed to evaluate the expression of collagenase mRNA expression, and 18S ribosomal RNA was used as a loading control **(Panel A)**. The ratios of collagenase mRNA expression/18S ribosomal RNA of each autoradiogram were then determined and the mean +/- SE was calculated and presented in **Panel B**. The p-value (<0.05) for collagenase mRNA expression between treated and untreated fibroblasts is considered to be significant (* symbol). Statistical evaluation between stratifin and insulin treated samples showed very significant differences (**$p<0.01$, n=3) while differences between insulin and stratifin plus insulin treated samples was significant (***$p<0.05$, n=3).

Figure 3.8.5. Northern analysis of collagenase mRNA expression of dermal fibroblasts treated for various times with stratifin (2.5 μg/ml) and insulin (10 mU/ml).

Autoradiogram of the Northern analysis of total RNA extracted from confluent normal dermal fibroblast cultures in DMEM plus 2% FBS exposed to stratifin alone (S), insulin alone (I), or both stratifin and insulin (S+I) for 0, 12, 24, 36, and 48 hours. Total RNA (10μg/lane) was electrophoresed on 1% agarose gels, transferred to nitrocellulose, and hybridized with collagenase cDNA probe (**Panel A**). The same blot was re-hybridized with cDNA specific for 18S ribosomal RNA and was used as a RNA loading control. Quantitative densitometry of the ratio of collagenase mRNA expression/18S ribosomal RNA with various treatments is shown as a function of time (hr) **(Panel B).**

3.8 Figures


A
Stratifin - + - + - +
50/50 50/50 D2 D2 D10 D10
collagenase
18S
B
2%FBS - + + + - -
high [Ca2+] - + - - + +
Stratifin - + - + - +
50/50 D2 50/50 50/50 50/50 50/50
collagenase
18S

Note: 50/50 = 50% DMEM & 50% KSFM
D2 = DMEM + 2% FBS
D10 = DMEM + 10% FBS

Figure 3.8.1 (Y. Marcoux generated Fig. 1a and I generated Fig. 1b)

Note:
50/50 = 50% DMEM & 50% KSFM
70/30 = 70% DMEM & 30% KSFM
90/10 = 90% DMEM & 10% KSFM

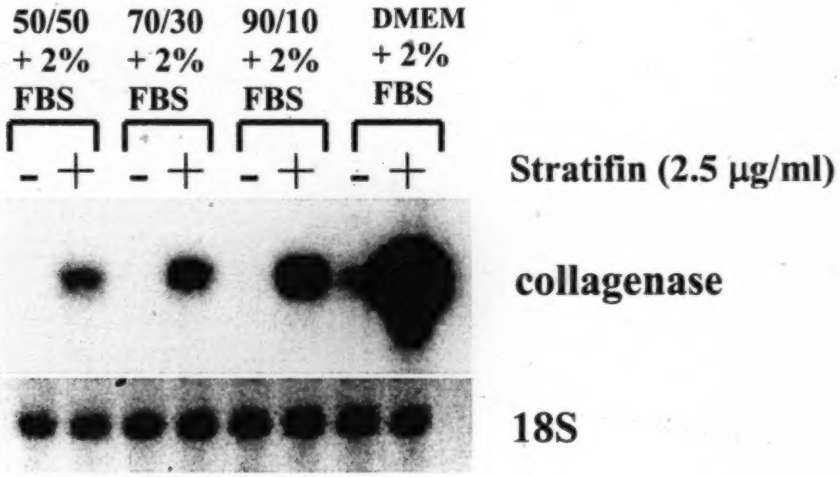
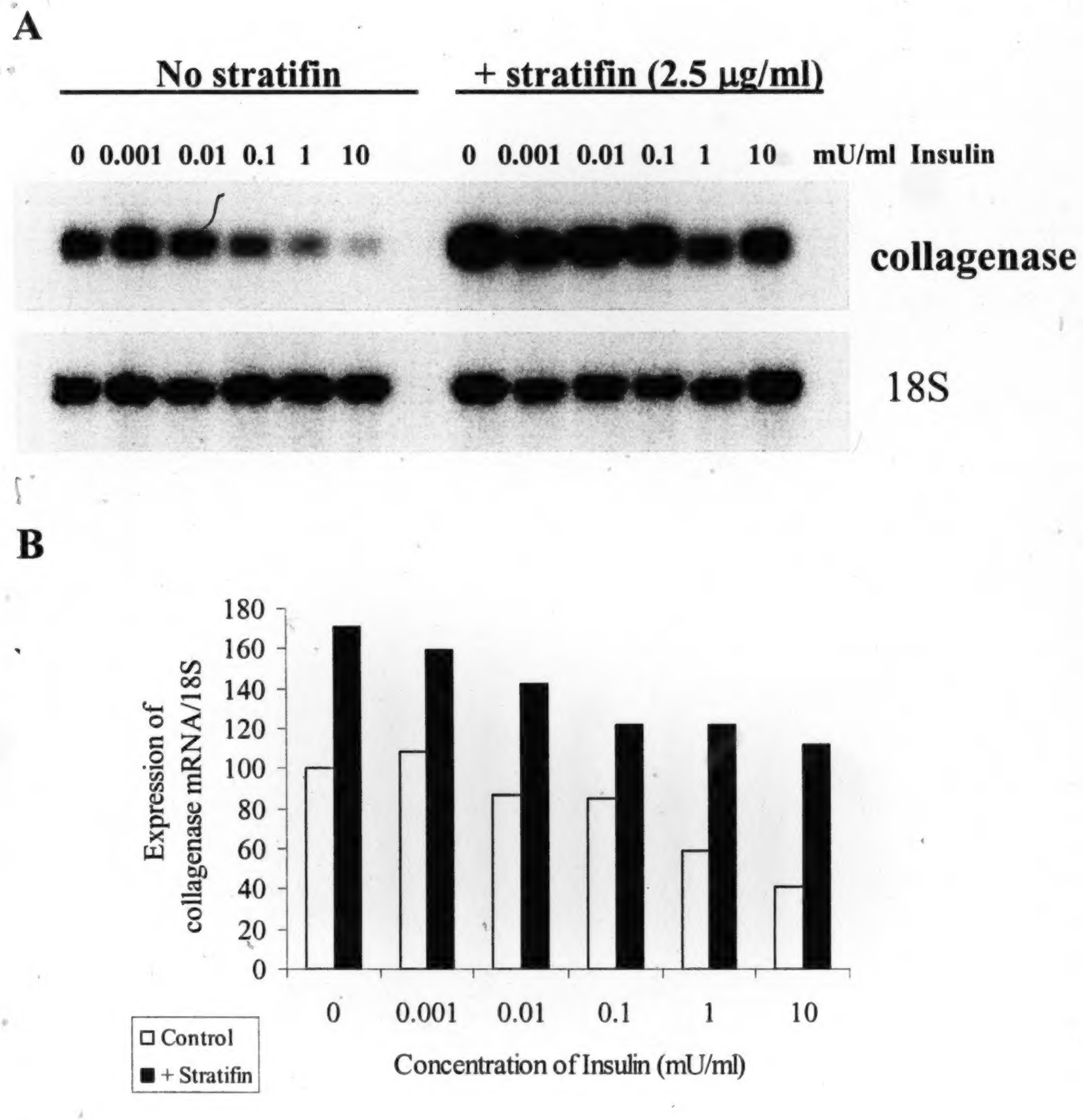

50/50 + 2% FBS
70/30 + 2% FBS
90/10 + 2% FBS
DMEM + 2% FBS
- + - + - + - +
Stratifin (2.5 μg/ml)
collagenase
18S

Figure 3.8.2 (I generated this figure)



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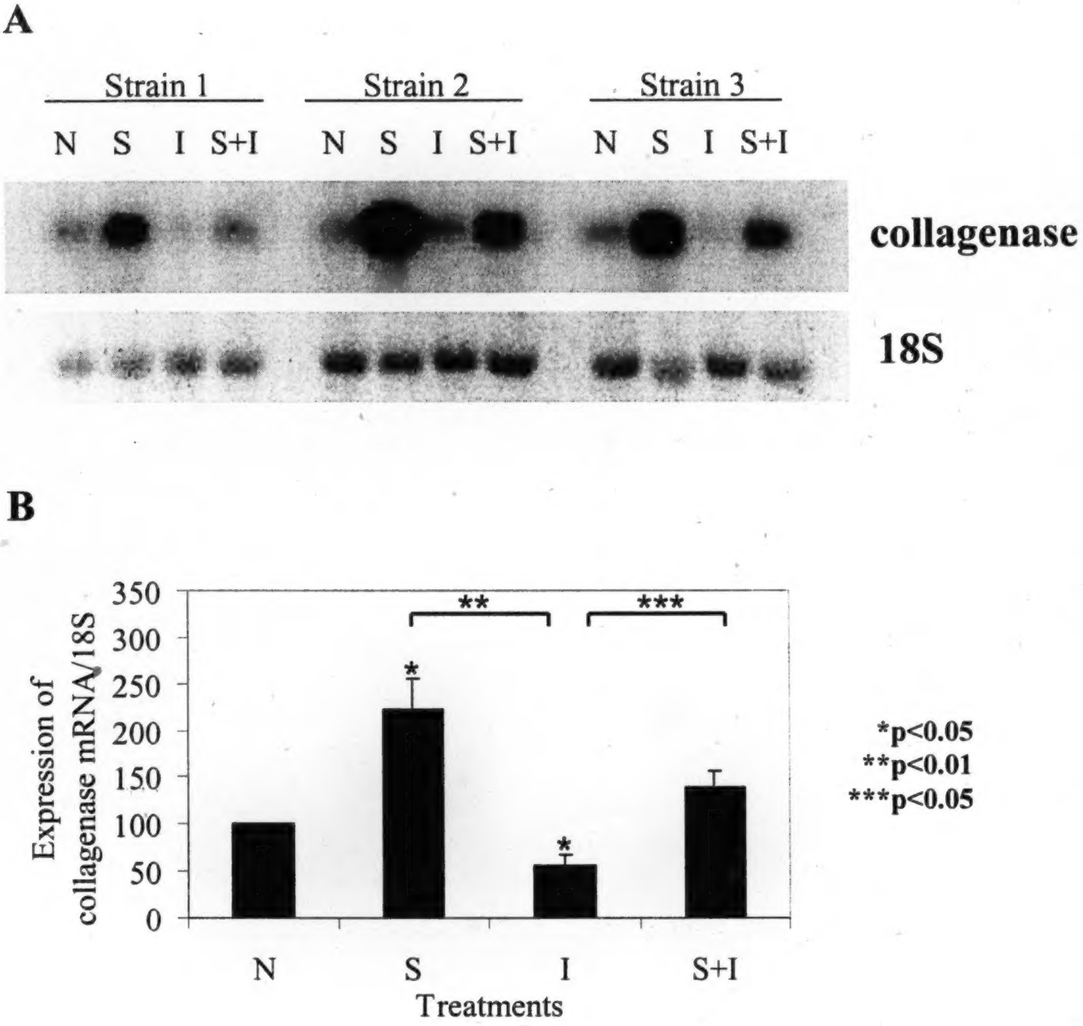

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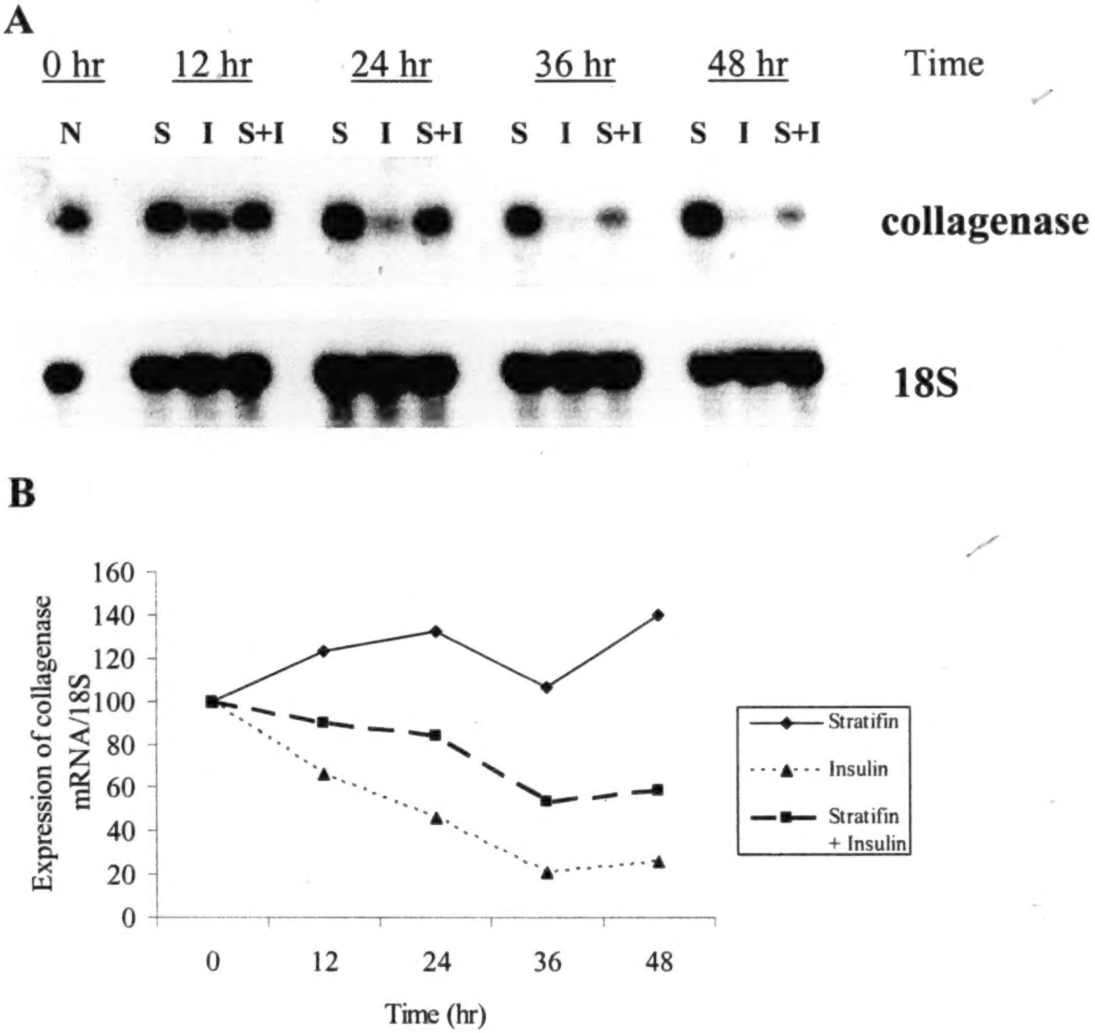

Figure 3.8.5 (I generated this figure)

CHAPTER 4:

STRATIFIN-INDUCED MMP-1 IN FIBROBLASTS IS MEDIATED BY *c-fos* AND p38 MAPK ACTIVATION

*A version of this chapter has been submitted for publication. E. Lam *et al*, 2004. Journal of Investigative Dermatology.

4.1 ABSTRACT

Various studies on stratifin have characterized its function as an intracellular protein and its extracellular function has not been explored until recently by our research group. Ghahary *et al* (2004) have demonstrated that keratinocyte-derived stratifin, also known as 14-3-3 σ protein, stimulates MMP-1 expression in dermal fibroblasts. In this study, I showed that 14-3-3 σ protein activates fibroblast MMP-1 mRNA and protein levels through p38 MAPK. My data indicated that treatment of dermal fibroblasts with 14-3-3 σ resulted in rapid and transient up-regulation of *c-jun* and *c-fos* mRNA levels. I also demonstrated that SB203580, a specific inhibitor of p38 MAPK activity, inhibited the activation of fibroblast MMP-1 mRNA expression by 14-3-3 σ. Subsequently, Western blot analysis revealed rapid and transient phosphorylation of p38 at 90 min after 14-3-3 σ stimulation and this was decreased to approximately 50% of the maximum value by 120 min. Stratifin was demonstrated to increase MMP-1 protein levels starting at 4 hr and reaching its peak at 12-24 hours. Furthermore, SB203580 significantly blocked the 14-3-3 σ induction of MMP-1 protein levels (***$p<0.005$, $n=3$). Microarray analysis of 14-3-3 σ treated fibroblasts showed an increase in Elk4/Sap1 mRNA expression. Array results were confirmed by RT-PCR and Northern blot analysis of Elk4/Sap1 mRNA expression. My results indicate that 14-3-3 σ markedly increase Elk4/Sap1 mRNA expression in a time dependent fashion. In conclusion, stratifin stimulates fibroblast MMP-1 levels through the activation of *c-fos* and p38 mitogen-activated protein kinase pathway.

4.2 INTRODUCTION

The matrix metalloproteinase (MMP) family consists of 25 zinc- and calcium-dependent proteinases in mammalian system. According to their substrate specificity, primary structure and cellular localization, MMPs are subdivided into five different classes: collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs (1). The basal expression of most MMPs in cultured cells is low, and these enzymes are transcriptionally induced by a variety of growth factors, cytokines, and tumor promoters (2-5). Since MMPs are involved in extracellular matrix (ECM) turnover and connective tissue remodeling, an imbalance in expression of MMPs has been implicated in a number of pathological conditions such as dermal fibrosis (6), rheumatoid arthritis, atherosclerosis, pulmonary emphysema and tumor invasion and metasis (2, 7).

Collagenase (MMP-1) is the principal fibroblast-derived secreted proteinase capable of degrading native fibrillar collagens of types I, II, III, and V. MMP-1 plays an important role in the remodeling of collagenous connective tissues in various physiological and pathological situations. A single activator protein-1 (AP-1) element which is located at –65 to –72 in the promoter region of human MMP-1 gene plays an essential role in the activation of MMP-1 gene transcription in response to a variety of extracellular signals (8). Adjacent to the AP-1 binding site is the E twenty-six (ETS) regulatory *cis*-element which also plays a role in the regulation of AP-1 dependent MMP-1 gene transcription (9, 10).

The expression and the activity of AP-1 and ETS transcription factors are induced by mitogen activated protein kinase (MAPK) in response to extracellular stimuli (11, 12). MAPKs, a family of eukaryotic serine/threonine protein kinases widely conserved among eukaryotes, play a role in the regulation of many cellular processes such as cell proliferation, migration and differentiation (13). There are presently three mammalian MAPK pathways characterized in detail: extracellular signal-regulated kinase-1/2 (ERK1/2), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 MAPK (8). While the p38 and JNK/SAPK pathways are activated by cellular stress, such as UV light, osmotic shock and inflammatory cytokines, the ERK1/2 pathway is activated by mitogenic growth factors via Ras and by phorbol esters via protein kinase C (14).

Among the MAPK subgroups, both ERK1/2 (15) and p38 MAPK are known to regulate MMP-1 gene expression. More specifically, the upregulation of MMP-1 gene expression by p38 MAPK is activated by a variety of stimuli including interleukin-1 (15, 16), C2 ceramide (17), tumor necrosis factor (15), extracellular MMP inducer (18) and okadaic acid (19).

In a previous study, Ghahary *et al* (2004) isolated a keratinocyte-derived anti-fibrogenic factor (KDAF) from keratinocyte-conditioned medium and subsequently identified it as the extracellular form of stratifin, also known as 14-3-3 σ, with potent MMP-1 stimulatory effects in dermal fibroblasts (20). However, the molecular mechanism by which 14-3-3 σ induces MMP-1 expression in fibroblasts is unknown. Therefore, the purpose of this present study was to identify signaling pathway elements mediating 14-3-3 σ stimulation of fibroblast MMP-1 expression by examining the three distinct MAPK pathways: ERK1/2, JNK, and p38. I hypothesize that 14-3-3 σ induces MMP-1 expression by one or more of the MAPK kinase pathways. In this study, I show that 14-3-3 σ protein increases the MMP-1 mRNA and protein levels in fibroblasts by *c-fos* and p38 MAP kinase activation.

4.3 MATERIALS AND METHODS

Fibroblast cell culture:

Cultures of human dermal fibroblasts were established as described previously (21). In brief, punch biopsy samples were prepared from human adult dermal fibroblasts. The tissue was collected in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, New York) plus 10% FBS supplemented with antibiotic-antimycotic preparation (100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B) (Gibco). Specimens were dissected and minced into small pieces less than 0.5 mm in diameter, washed six times with DMEM, and distributed into 60 X 15-mm culture dishes (Corning Inc., Corning, NY), four pieces per dish. After 4 weeks of incubation, the cells were released from dishes by brief treatment with 0.1% trypsin (Life technologies Inc., Gaithersburg, MD) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) in PBS (pH 7.4) and reseeded into 75-cm^2 flasks (Corning Inc). Upon reaching confluence, the cells were subcultured at a ratio of 1:6 by trypsinization. Fibroblasts from passages 3-8 were used for this study.

Preparation of human recombinant stratifin (14-3-3 σ):

Procedures of human recombinant 14-3-3 σ protein preparation were established as described previously (20). Briefly, the cDNA of 14-3-3 σ from human keratinocytes was cloned into pGEX-6P-1 expression vector (Amersham/Pharmacia Biotech) and transformed into protein expressing bacteria, BL-21 (DE3) (Novagene). A single positive clone was grown in 100 ml of LB medium containing 50 μg/ml of ampicillin for 4-6 hours at 29 °C until an OD_{600nm} of 0.4-0.6 was reached. Bacteria were then diluted to 1:10 with LB medium plus 0.1 mM IPTG for 12 hrs. To purify the protein, bacteria were centrifuged and lysed with 50mM Tris-HCl (pH 7.4) containing 10mM EDTA, 5mM EGTA, protease inhibitor cocktail (Sigma), 1% Triton X-100, and 0.5% IGEPAL CA630. Cell lysate was passed through a Glutathione Sepharose 4B affinity column and subsequently washed with PBS containing 0.1% Triton X-100 until an OD_{280nm} reached zero. GST-fused 14-3-3 σ was digested using PreScission protease according to manufacturer's procedure (Amersham/Pharmacia Biotech). GST-free 14-3-3 σ was then eluted, dialysed against PBS and then concentrated with Centricon (Millipore). The sequence of protein was validated on a Bruker REFLEX III mass spectrometer (Bremen/Leipzig, Germany) using MALDI in positive ion mode.

Treatment of fibroblasts:

Fibroblasts were seeded into 60 X 15-mm petri dishes (Corning Inc.). For each experiment, confluent fibroblasts were rinsed with phosphate-buffered saline (PBS) before DMEM plus 2% FBS was added. At this time, 2.5 μg/ml of 14-3-3 σ was incubated for 24 hrs to stimulate collagenase mRNA expression. Based on previous experiments, the stratifin protein concentration of 2.5 μg/ml was the optimal dose for the stimulation of MMP-1 mRNA expression in dermal fibroblasts for 24 hr incubation period. For the inhibition of ERK1/2, p38, and JNK MAPK, 10μM of specific inhibitors against each kinase (PD98059, SB203580 and SP600125, respectively) were added 1 h before stimulation. MEK1/2 inhibitor PD98059, p38 inhibitor SB203580, and JNK inhibitor SP600125 were purchased from Calbiochem (San Diego, CA). For Western blot analysis of total and phosphorylated forms of p38 MAPK protein levels, confluent fibroblasts were switched to serum free media for 24 hours before treatment in order to reduce the background phosphorylation levels of MAPKs due to serum.

RNA isolation and Northern blot analysis:

Fibroblasts were harvested with 400 μl of 4 M guanidium isothiocyanate (GITC) solution and total RNA from each group was isolated by the acid-guanidium-phenol-chloroform method (22). Total RNA from each individual fibroblast culture was then separated by electrophoresis (10μg per lane) on a 1% agarose gel containing 2.2 M formaldehyde and was blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California). The blots were baked for 2 hr at 80°C under vacuum and prehybridized for 4 hr at 45 °C in a prehybridization solution. Hybridization was performed at 45 °C in the same solution, using MMP-1, Elk4/Sap1, or 18S ribosomal RNA cDNA probes. The cDNA probes were labeled with P-α^{32}-dCTP by nick translation. The filters were washed initially at room temperature with

2X sodium citrate/sodium chloride buffers and 0.1% sodium dodecylsulfate for 1 hr and finally washed for 20 min at 65 °C in 0.1X sodium citrate/sodium chloride buffer and 0.1% sodium dodecylsulfate. Autoradiography was performed by exposing Kodak X-Omat film to nitrocellulose filters at -80 °C in the presence of an intensifying screen. Quantitative analysis of autoradiographs was accomplished by densitometry. Each experiment was performed at least twice to ensure reproducibility of the results.

The cDNA probes for MMP-1, *c-fos*, *c-jun*, and 18S ribosomal RNA were obtained from the American Type Culture Collection (Rockville, MD). The cDNA probe for Elk4/Sap1 was obtained by extracting fibroblast total RNA and was amplified by RT-PCR. The PCR product was then purified and ligated into a pGEX-6P-1 vector (Amersham Pharmacia Biotech). The ligated products were then transformed to competent DH5α cells with the regular heat-shock transformation method. Positive clones were identified by the size of restriction enzyme-digested products. DNA sequence was confirmed by fluorescence dNTP sequence analysis.

Cell Protein Lysate Extraction and Western Blot Analysis:

Cell extracts were prepared from 1.5X10^6 cells in lysis buffer (20-mM Tris-HCL [pH 7.5], 150-mM NaCl, 2-mM EDTA, 1% Triton X-100, 10% Glycerol, 100-mM PMSF, protease inhibitor cocktail, and Na_3VO_4 to prevent dephosphorylation of p38 [Sigma Chemicals]). Extracts were centrifuged at 13,000 RPM for 10 minutes. The total protein contents of cell lysate were determined by BSA assay (Pierce, Rockford, IL, USA). Equivalent amounts of fibroblasts extract (100 µg/lane) were resolved by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred onto PVDF membranes (Millipore Corporation).

For the detection of p38 MAPKs, rabbit IgG antibodies to total and phosphorylated forms of p38 were obtained from Cell Signaling (Beverly, MA). The membranes were blocked in 5% skim milk powder in PBS 0.1% Tween 20 for 2 hrs at room temperature. Membranes were then treated overnight at 4°C with antibodies to total or phosphorylated forms of the signalling intermediates (1:1000 dilutions) in PBS 0.1% Tween 20 plus 5% BSA. Following several rinses in PBS 0.1% Tween 20 for 30 mins, the membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-rabbit IgG (Bio-Rad) (1:2500 dilution). Immunoreactive proteins were then visualized using ECL + plus Western blotting detection system (Amersham Biosciences, Buckinghamshire, England).

For the detection of collagenase and β-actin protein levels, cell lysates (40 μg/lane) from each treatment were subjected to SDS-PAGE analysis with 12% (wt/vol) acrylamide gel, and electrotransferred onto PVDF membranes (Millipore Corporation). The membranes were blocked in 5% skim milk powder in PBS 0.1% Tween 20 overnight. Immunoblotting was performed using 2 μg/ml of mouse anti-human MMP-1 monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or 2 μg/ml of mouse anti-human β-actin monoclonal antibody (Santa Cruz Biotechnology, Inc.). The membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) antibodies (1:2500 dilution).

Immunoreactive proteins were then visualized using ECL + plus Western blotting detection system (Amersham Biosciences, Buckinghamshire, England).

Microarray Experiments

To examine whether 14-3-3 σ induced other genes involved in the MAPK signalling pathway, GEArray gene expression array systems were purchased from SuperArray Bioscience Corporation (Bethesda, MD). Each GEArray Q Series Human MAP Kinase Signalling pathways gene array consists of 96 genes known to be involved in MAPK pathways as well as control sequences (PUC18 as negative control; β-actin, cyclophilin A, and glyceraldehydes 3 phosphate dehydrogenase [GADPH] for loading). Using different arrays, we compared the gene expressions of untreated and 14-3-3 σ treated fibroblasts. For these experiments, total cellular RNA was isolated by a modification of the guanidinium isothiocyanate technique. Integrity of RNA was assessed by visualization of ethidium bromide-stained gels. The microarrays were employed according to the manufacturer's instructions. In brief, using reagents provided, cDNA was prepared from total RNA by reverse transcription with MMLV reverse transcriptase, biotinylated with Biotin-16-dUTP (Roche, Indianapolis, IN), then hybridized under precisely specified conditions to a positively charged nylon membrane containing the arrayed DNA. The arrays were visualized using the chemiluminescent detection system purchased from GEArray (Bethesda, MD). Loading was adjusted based on intensity of hybridization signals to the housekeeping gene, cyclophilin A, then gene expression was quantified by scanning densitometry.

Reverse Transcriptase-Polymerase Chain Reaction

cDNA was synthesized from total RNA with oligo (dT) primer and MMLV reverse transcriptase (Invitrogen Life Technologies). Samples were incubated at 42 °C for 60 minutes, and the reaction was terminated by heating at 70 °C for 15 minutes, followed by rapid chilling on ice for 2 minutes. Polymerase chain reaction (PCR) was carried out using human Elk4/Sap1 primers (sense: 5'-GCGAATTCGAAGCCTCAGAACAAGCA-3'; antisense: 5'-GCCTCGAGTAAGAGAAGCTGTAGGGAGA-3'; size about 1000bp) and β-actin primers (sense: 5'-CCCCCATGCCATCCTGCGTCTG-3'; antisense: 5' -CATGATGGAGTTGAAGGTAGTTT-3'; size about 336 bp), respectively. The housekeeping β-actin mRNA was used as a loading control. Polymerase chain reaction was conducted with 25 cycles for β-actin and 40 cycles for Elk4/Sap1. PCR products were separated by electrophoresis in 1% agarose gels, stained with ethidium bromide and visualized under ultraviolet light.

Statistical Analysis

Autoradiograms of Northern analysis were quantified with densitometry and data expressed as a relative OD value. The statistical significance of differences in collagenase mRNA expression between treated and untreated dermal fibroblasts was tested with Student's unpaired two-tailed *t*-test; p values < 0.05 and < 0.01 were considered significant and very significant, respectively.

4.4 RESULTS

Activation of c-jun, c-fos, and MMP-1 mRNA expression by 14-3-3 σ

The enhancement of human MMP-1 gene transcription involves activation of AP-1 element located around -70 in the 5'-flanking regulatory region of the genes (8). Moreover, the induction of expression of the principal components of the AP-1 dimers, c-Jun and c-Fos, is mediated by distinct MAPK pathways (8, 12). As shown in Figure 4.8.1A, treatment of dermal fibroblasts with 14-3-3 σ resulted in rapid and transient up-regulation of *c-jun* and *c-fos* mRNA levels. MMP-1 mRNA levels were induced at 4 hours of 14-3-3 σ treatment. These observations show that 14-3-3 σ induction of MMP-1 gene transcription is likely to be mediated through the AP-1 binding element. Densitometry results revealed that stratifin markedly increases *c-jun* and *c-fos* mRNA/18S ratio at 1 hr followed by dramatic induction of MMP-1 mRNA/18S ratio at 4 hrs in fibroblasts (Figure 4.8.1B).

Induction of MMP-1 Expression by 14-3-3 σ is mediated by p38

To determine the possible signal pathway in which 14-3-3 σ proteins increase the expression of MMP-1, we explored the three distinct MAPK pathways in which fibroblast MMP-1 expression is regulated: a) *ERK1/2* (Raf=>MEK1/2=>ERK1/2); b) *JNK/SAPK* (MEK kinase 1-3=>MEK kinase 4 and 7=>JNK/SAPK); c) *p38 MAPK* (MAPK kinase kinase=>MAPK kinase 3 and 6=>p38). We examined the role of the specific MAPK pathways in the 14-3-3 σ-elicited activation of collagenase mRNA expression in dermal fibroblasts by blocking MEK1/2 and p38 MAPK pathways with chemical inhibitors previously shown to block MMP-1 gene activation by diverse stimuli (16, 17, 19). As seen in Figure 4.8.2A, activation of fibroblast MMP-1 mRNA expression by 14-3-3 σ was potently inhibited by SB203580, a specific inhibitor of p38 activity. In parallel, blocking the activity of MEK1/2 and JNK by specific inhibitors PD98059 and SP600125, respectively, had no marked effect on the activation of fibroblast MMP-1 expression by 14-3-3 σ.

In the same experimental setting, fibroblasts were treated with various concentrations (1, 5, 10, 50 μM) of SB203580 in the presence of 2.5 μg/ml of stratifin for 24 hours (Figure 4.8.2B). The results show that SB203580 inhibits the efficacy of stratifin to increase MMP-1 mRNA expression and this inhibitory effect is proportional to the amounts of inhibitor used. Stimulation of MMP-1 by stratifin was completely abrogated at 50 μM of SB203580. This finding suggests that stratifin may function through the p38 MAPK pathway.

p38 Phosphorylation in fibroblasts stimulated by 14-3-3 σ

Human skin fibroblasts were treated with 14-3-3 σ (2.5 μg/ml) for different periods of time and the activation of p38 was determined by Western blot analysis using antibodies against activated forms of p38 MAPK. Figure 4.8.3A demonstrates changes in p38 phosphorylation at 15, 30, 60, 90, 120, 240 min in fibroblasts exposed to 14-3-3 σ. Rapid and transient phosphorylation of p38 was observed at 90 min after stimulation and decreased to approximately 50% of the maximum value by 120 min.

In the next set of experiments, the effects of SB203580 (10μM) on p38 phosphorylation induced by 14-3-3 σ were assessed (Figure 4.8.3B). Consistent with the findings shown in Panel A, fibroblasts treated with 14-3-3 σ for 90 min increased p38 phosphorylation and this effect was enhanced with the treatment of SB203580. Densitometry results from three separate experiments in Figure 3C indicate that in the presence of both 14-3-3 σ and SB203580, the level of phosphorylation was very significantly increased (**p=0.0001, n=3). The data shows the consequence of inhibiting downstream activity of p38 MAPK, thereby precluding the effects of phosphatases normally activated after MAPK phosphorylation to dephosphorylate and inactivate the signal cascade. Therefore, the effects of SB203580 prolonged the phosphorylated state of p38 MAPK in the absence (*p<0.0001, n=3) as well as in the presence of 14-3-3 σ treatment (*p<0.0001, n=3). However, the increase of p38 MAPK phosphorylation induced by the inhibitor was more dramatic when 14-3-3 σ was added (***p<0.005, n=3). These data support the effects of SB203580 on p38 phosphorylation induced by keratinocyte-conditioned medium in which the levels of phosphorylation was increased in the presence of inhibitor in addition to stimuli of MMP-1 gene expression (23).

The levels of MMP-1 protein in fibroblasts is stimulated by 14-3-3 σ via p38 MAPK

Fibroblasts were treated with 14-3-3 σ (2.5 μg/ml) for different periods of time and MMP-1 protein levels were determined by Western blot analysis. The levels of β-actin were also determined in the same samples by Western blot analysis for loading controls. Figure 4.8.4A shows the changes in MMP-1 intracellular protein levels at 2, 4, 6, 12, and 24 hr in fibroblasts stimulated by 14-3-3 σ. The results reveal an increase in collagenase protein levels starting at 4 hr and reaching its peak at 12-24 hours.

In the same experimental setting, the effects of SB203580 on the 14-3-3 σ-induced collagenase levels were assessed on Western blot analysis (Figure 4.8.4B). Fibroblasts were treated for 24 hours with 2.5 μg/ml of 14-3-3 σ, 10μM of SB203580 or a combination of both. Once again, our data indicate that MMP-1 intracellular protein levels are significantly increased after 24 hours treatment with 14-3-3 σ (*$p<0.005$, n=3). As expected, p38 MAPK inhibitor blocked the 14-3-3 σ induction of MMP-1 protein levels. In Figure 4.8.4C, densitometry results from three separate experiments revealed very significant differences in collagenase levels between 14-3-3 σ and 14-3-3 σ plus SB203580 treated samples (***$p<0.005$, n=3).

Microarray analysis of MAPK signaling pathway genes of 14-3-3 σ treated fibroblasts

Figure 4.8.5A shows array results of MAPK signaling pathway gene expression in untreated and 14-3-3 σ treated fibroblasts. Expression is normalized for cyclophilin A (PPIA). Compared to untreated samples, fibroblasts treated with 14-3-3 σ reveal an increase Elk4/Sap1a and autotoxin (ENPP2) by 3.5 and 4.75 fold, respectively (Panel B). Interestingly, Elk4/Sap1a is a transcription factor with the ability to form a ternary nucleoprotein complex with the serum response factor (SRF) over the serum response element (SRE) (24). The SRE is constitutively occupied by a protein complex comprised of SRF and ternary complex factor (TCF) and pivotal for transcriptional up-regulation of the c-fos proto-oncogene, whose gene product is an essential AP-1 component of modulation in MMP-1 gene expression. To confirm the array results, Northern analysis of Elk4/Sap1 mRNA and 18S rRNA expression was performed on total RNA samples extracted from fibroblasts that were treated with 2.5 μg/ml of 14-3-3 σ for the various time intervals. Figure 4.8.6A demonstrates changes in Elk4/Sap1 mRNA expression at 0, 0.5, 2, 4, 6, 12, 24 hours in fibroblasts exposed to 14-3-3 σ. Up-regulation of Elk4/Sap1 expression was observed at 0.5 hour after stimulation and peaked in intensity at 4 hours and subsequently decreased to approximately 50% of the maximum value by 6 hours. This finding reveals that 14-3-3 σ markedly increases Elk4/Sap1 mRNA expression in a time dependent fashion in fibroblasts. Confirmation of array results by RT-PCR for Elk4/Sap1 is shown in Figure 4.8.6B. Total RNA samples were extracted from fibroblasts with stratifin treatment for 0, 0.5, 2, 4, 6, 12 hours. Our results show that stratifin up-regulates Elk4/Sap1 gene expression and this peaks at 4-6 hours post treatment. RT-PCR was performed for the same samples with β-actin primers for loading control.

4.5 DISCUSSION

MAPKs mediate extracellular signals, which regulate cell growth, survival, differentiation, and death. At present, three distinct MAPK pathways are known in detail: ERK1/2, JNK/SAPK, and p38 MAPK (8). Specific upstream dual-specificity kinases (MAPK kinases) are able to phosphorylate the conserved threonine and tyrosine residues of MAPKs, which in turn phosphorylate and activate nuclear protein kinases such as MAPK-activated protein kinases 1, -2, -3 or transcription factors including c-Jun, Elk-1, and activating transcription factor-2 (8). A series of MAP kinase nuclear substrates including those directly involved in MMP-1 transcriptional regulation such as the gene products of *fos* and *jun* oncogenes compose the AP-1 transcriptional factor (25-28). AP-1 is activated by a variety of signaling cascades, some of which are p38-dependent (29).

In this present study, recombinant 14-3-3 σ protein was added to fibroblasts to induce MMP-1 mRNA expression and this was shown to be mediated by p38 MAP kinase. In addition, blocking the p38 pathway with SB203580 inhibits the up-regulation of endogenous MMP-1 gene expression by exogenous 14-3-3 σ. These observations are in accordance with the previous study performed by Moon *et al* (2000) in which fibroblasts exposed to keratinocyte-conditioned medium induced MMP-1 mRNA expression through p38 MAPK pathway (23). They also demonstrated that p38 signalling inhibitor (SB203580) blocked MMP-1 elaboration induced by keratinocyte-conditioned medium (23).

Other studies have also identified the expression of fibroblast MMP-1 to be dependent on p38 MAPK expression (15, 18, 19). Moreover, p38 MAPK has been implicated to mediate the expression of several other metalloproteinase genes important in extracellular matrix remodelling including, MMP-9 (30) and MMP-3 (31). Originally described to be a stress-activated enzyme (32), p38 has also been shown to have downstream effects on actin filament rearrangement (33), transcription factor activation (33), and matrix degradation (18, 19, 30, 31). Based on these observations, p38 may be an important MAP kinase in the general process of tissue remodelling. In addition, specific inhibitors of the p38 MAPK pathway may eventually serve as novel therapeutic agents for inhibiting degradation of collagenous extracellular matrix.

According to my microarray results, 14-3-3 σ up-regulates Elk4/Sap1a transcription factor (Figure 5). This finding was further confirmed by Northern blot analysis and RT-PCR (Figure 6). The ETS domain transcription factor, Sap1, belongs to the family of ternary complex factors (TCF) and has high homology with Elk-1 (24). Sap1 contains a MAP kinase docking domain that exhibits strong sequence similarity to the Elk-1 D-domain (34). Although Sap1 has been shown to be able to act as a target of the ERK, JNK, and p38 MAPK families (35-38), Sap1 is preferentially phosphorylated by p38α MAPK on the homologous residues, Ser381 and Ser387, indicating that specificity determinants exist. Mutation of these sites to alanine severely reduces c-fos SRE-dependent transcription mediated by Sap1a and p38 MAPK (37). The kinase selectivity of Sap1 is determined by its D-domain (34). Sap1 requires DNA-bound SRF for ternary complex formation and makes extensive DNA contacts to the 5' side of SRF, but does not bind DNA autonomously. Moreover, Sap1 interacts with the serum response element (SRE) in the absence of serum response factor (SRF), although cooperative binding of Sap1 with SRF is observed (39). If this is the case, the presence of serum would be required for 14-3-3 σ to regulate *c-fos* transcription and thus induce MMP-1 gene expression. The results in our laboratory support this phenomenon since the 14-3-3 σ fails to increase MMP-1 gene expression in fibroblasts in the absence of serum (data not shown).

Another gene which 14-3-3 σ up-regulated in fibroblasts encodes Autotaxin (ATX), a glycoprotein that belongs to the nucleotide pyrophosphatase and phosphodiesterase (NPP) family of ectoenzymes (40). Originally thought to be an ecto-phosphodiesterase, ATX was recently found to possess lysophospholipase D (lyso-LPD) activity (41). The lyso-LPD activity of ATX can generate the bioactive mediators lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) from appropriate precursors (41, 42). Studies done by Lee *et al* (2000) have shown that LPA and S1P stimulate endothelial cell wound healing by increasing cell migration and proliferation (43). Moreover, these lipids modulate the synthesis and release of peptide growth factors involved in wound healing. It has been shown that LPA stimulates the secretion of transforming growth factor-β (TGF-β) in keratinocytes (44) and insulin-like growth factor II in human breast and ovarian cancer cells (45, 46). Further studies in our laboratory will determine whether ATX is involved in the coordinated events of wound healing.

In conclusion, I have characterized for the first time that 14-3-3 stimulates the signal pathway of the expression of fibroblast MMP-1 at the promoter, mRNA, and protein levels. My data suggests that the enhancement of fibroblast mRNA expression as well as protein levels by keratinocyte-derived 14-3-3 σ protein is mediated by p38 MAPK. Activated p38 MAPK is then able to phosphorylate and activate Elk4/Sap1a in response to 14-3-3 σ to induce c-fos gene transcription and subsequently form AP-1 components to modulate MMP-1 gene expression and intracellular protein levels. These data indicate that keratinocyte-derived 14-3-3 σ protein modulate fibroblast MMP-1 levels through the p38 mitogen-activated protein kinase pathway leading to altered biological functions of wound healing.

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4.7 Figure Legends

Figure 4.8.1. 14-3-3 σ activates the MMP-1 promoter via AP-1.

Confluent human skin fibroblasts in DMEM and 2% FBS were incubated with 14-3-3 σ (2.5 μg/ml) for 0, 0.5, 1, 2, 4, 6, 12, 24 hours. Total RNA (10μg/lane) was electrophoresed on 1% agarose gels, transferred to nitrocellulose, and hybridized with collagenase cDNA. Levels of MMP-1, *c-jun*, *c-fos* mRNA and 18S rRNA were determined by Northern blot hybridizations of total RNA (10 μg/ml).) Panel A. The same blots were re-hybridized with cDNA specific for 18S ribosomal RNA and were used as a RNA loading control. Quantitative densitometry of the ratio of collagenase mRNA expression/18S ribosomal RNA with various treatments is shown as a function of time (hr) (Panel B).

Figure 4.8.2. Induction of MMP-1 expression by 14-3-3 σ is mediated by p38 MAPK.

Fibroblasts in DMEM and 2% FBS were incubated with 10 μM of specific inhibitors against each MAPK kinase for 1 hr before stimulation 14-3-3 σ (2.5 μg/ml) for 24 hours (Panel A). In Panel B, fibroblasts were treated in DMEM plus 2% FBS with 1, 5, 10, 50 μM of SB203580 for a period of 48 hrs. For both sets of experiment, total RNA was extracted and Northern blot analysis was performed to determine the expression of collagenase. The same blot was re-hybridized with cDNA specific for 18S ribosomal RNA and was used as a RNA loading control.

Figure 4.8.3. 14-3-3 σ induces p38 MAPK phosphorylation in dermal fibroblasts.

Fibroblasts were treated with 14-3-3 σ (2.5 μg/ml) for various time intervals as indicated. The levels of activated p38 (*p-p38*) were determined by Western blot analysis using phospho-specific antibodies for p38 MAPK. The levels of total p38 were also determined in the same samples by Western blot analysis using specific antibodies. C6 cell extracts treated with anisomycin were used as positive control for phospho-p38 (Panel A). In Panel B, fibroblasts were treated with either 14-3-3 σ (2.5 μg/ml), SB203580 (10μM), or a combination of both. Fibroblasts were pretreated with SB203580 (10μM) for 1 hr before treatment with 14-3-3 σ. The membrane was immunoblotted using antibodies against total and phosphorylated forms of p38 MAPK and subsequently immunoblotted with β-actin antibody as loading control. Statistical evaluation between samples that were untreated and treated with 14-3-3 σ or SB203580 or a combination of both showed very significant differences ($^*p<0.0001$, n=3), while the difference between 14-3-3 σ and 14-3-3 σ plus SB203580 treated samples was very significant ($^{**}p=0.0001$, n=3). The differences between 14-3-3 σ and SB203580 treated samples as well as SB203580 and 14-3-3 σ plus SB203580 treated samples was very significant ($^{**}p=0.0001$, n=3, $^{***}p<0.005$, n=3, respectively) (Panel C).

Figure 4.8.4. Induction of intracellular MMP-1 protein levels by 14-3-3 σ is mediated by p38 MAPK.

Fibroblasts were treated with 14-3-3 σ (2.5 μg/ml) for different periods of time as indicated Panel A. The intracellular MMP-1 protein levels were determined by Western blot analysis using specific antibodies. The levels of β-actin were also determined in the same samples by Western blot analysis for loading controls. In Panel B, fibroblasts were treated for 24 hrs with either, 14-3-3 σ (2.5 μg/ml), SB203580 (10μM), or a combination of both. Fibroblasts were pretreated with SB203580 for 1 hr before treatment with 14-3-3 σ. β-actin is shown as loading controls.

Figure 4.8.5. Microarray analysis of mRNA extracted from 14-3-3 σ treated fibroblasts.

Fibroblasts were treated with 14-3-3 σ (2.5 μg/ml) for 90 min. Total mRNA was extracted and biotin-labeled cDNA were prepared followed by hybridization to the gene arrays according to the manufacturer's directions. Panel A shows the results of untreated and 14-3-3 σ treated samples of MAP Kinase signaling pathway gene array. PPIA expression is used as positive control as well as normalization and PUC18 for negative control while the highlighted box illustrates expression of Elk4/Sap1 and autotaxin (ENPP2). A key to gene coordinates is shown in Panel B as well as a table indicating the fold increase of Elk4/Sap1a and autotaxin from 14-3-3 σ treatment as compared to controls.

Figure 4.8.6. Confirmation of array results by Northern Analysis and RT-PCR of Elk4/Sap1.

Fibroblasts were treated with 14-3-3 σ (2.5 μg/ml) for different time intervals. The total RNA was extracted and Northern blot analysis was performed to determine the expression of Elk4/Sap1 (Panel A). The same blot was re-hybridized with cDNA specific for 18S ribosomal RNA and was used as a RNA loading control. In Panel B, RT-PCR with Elk4/Sap1 primers was performed from RNA samples extracted from stratifin treated fibroblasts. The same samples were used for RT-PCR with β-actin primers for loading control.

4.8 Figures

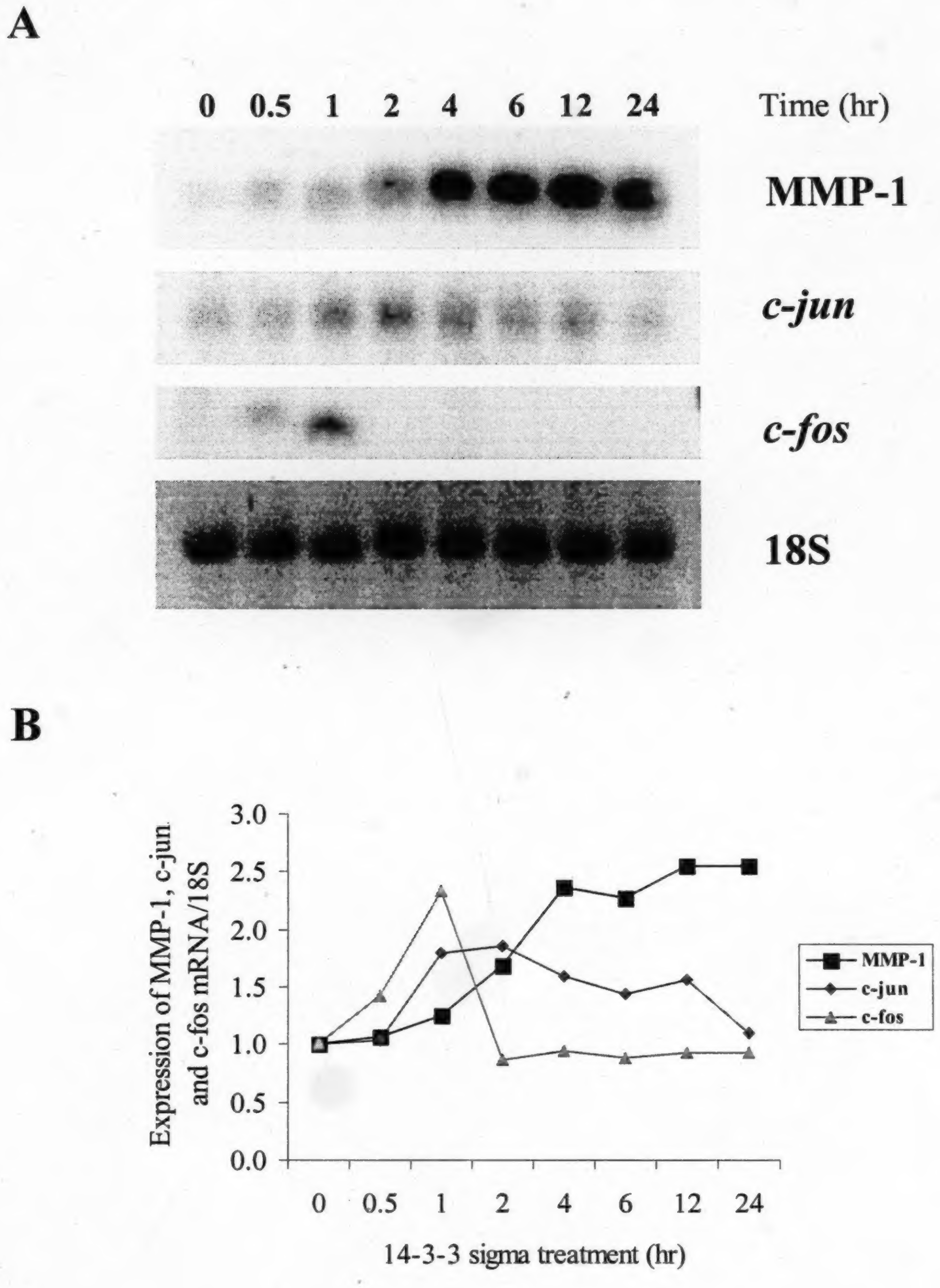
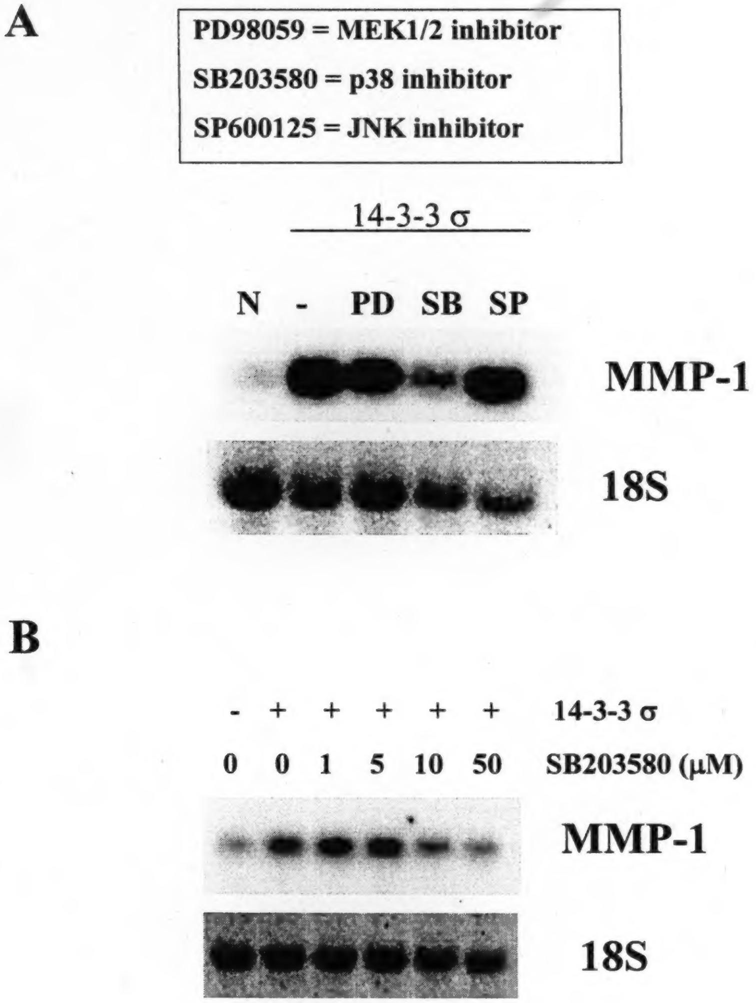


Figure 4.8.1 (I generated this figure)


A
PD98059 = MEK1/2 inhibitor
SB203580 = p38 inhibitor
SP600125 = JNK inhibitor
14-3-3 σ
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-
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18S
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- + + + + +
14-3-3 σ
0 0 1 5 10 50
SB203580 (μM)
MMP-1
18S

Figure 4.8.2 (I generated this figure)

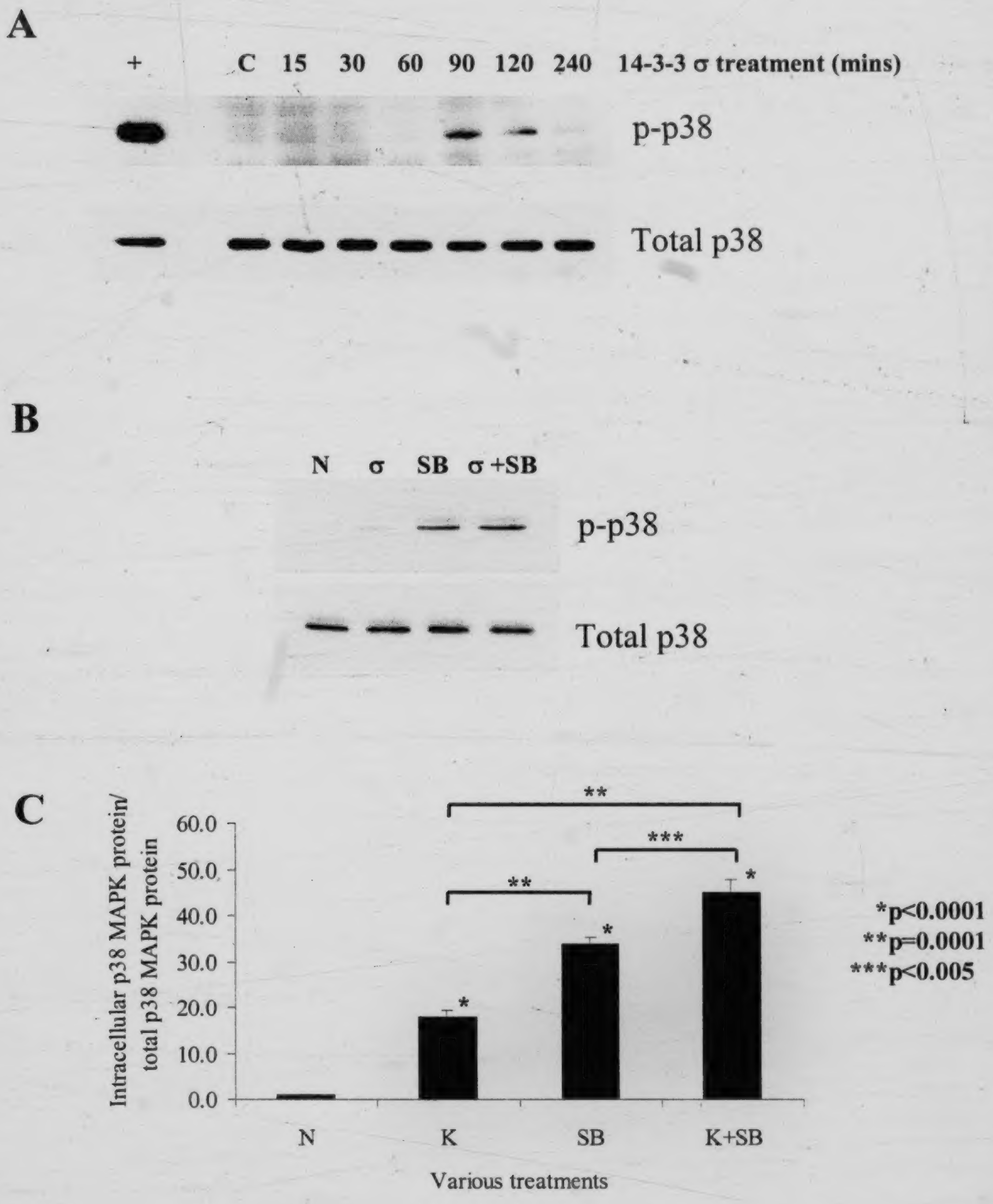


Figure 4.8.3 (I generated this figure)

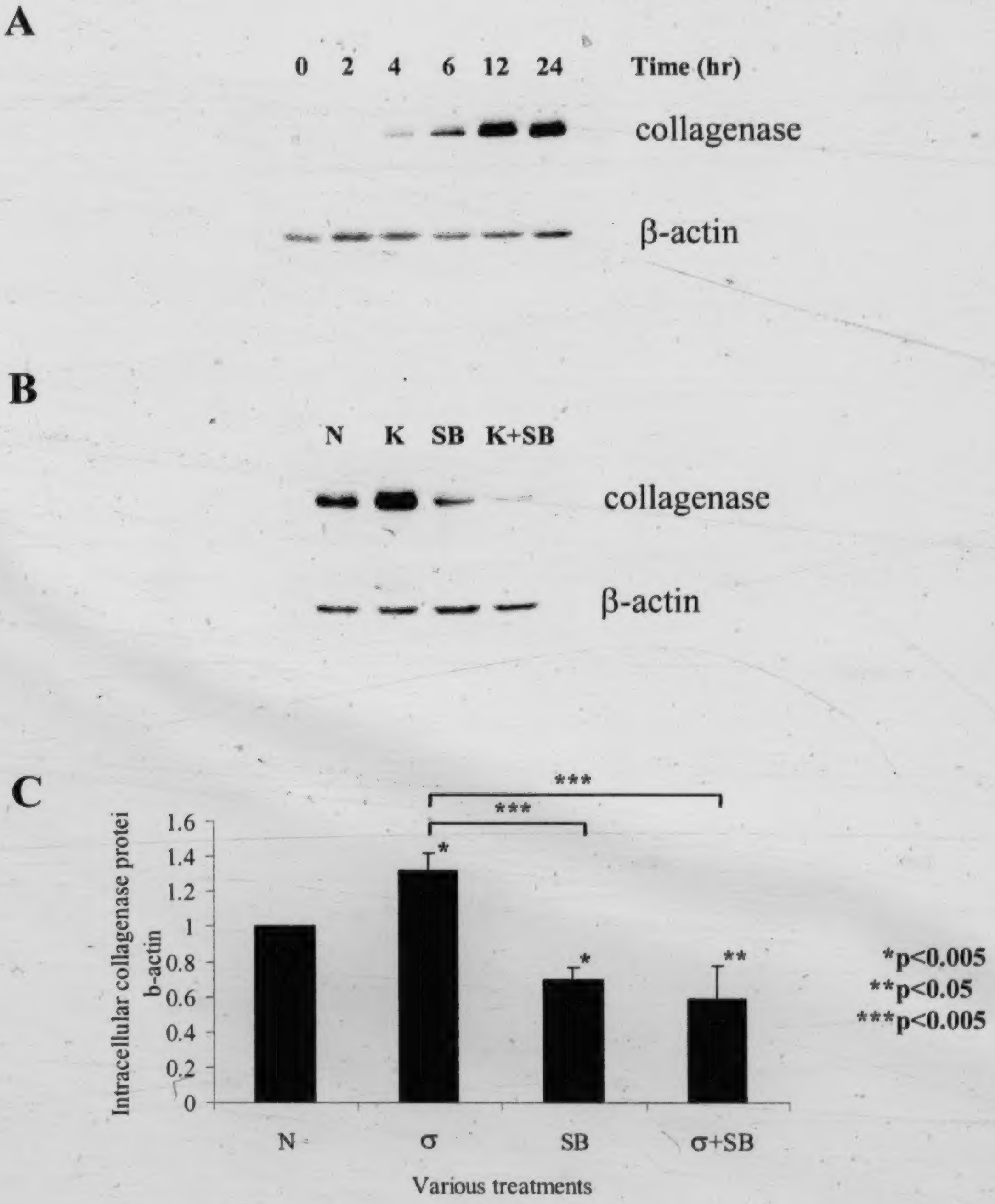
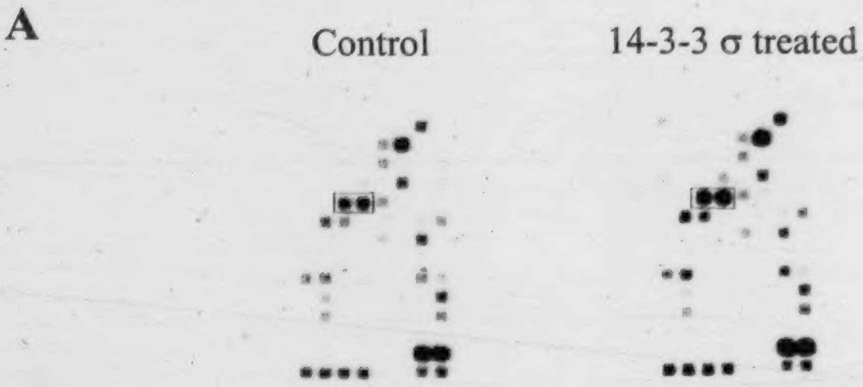
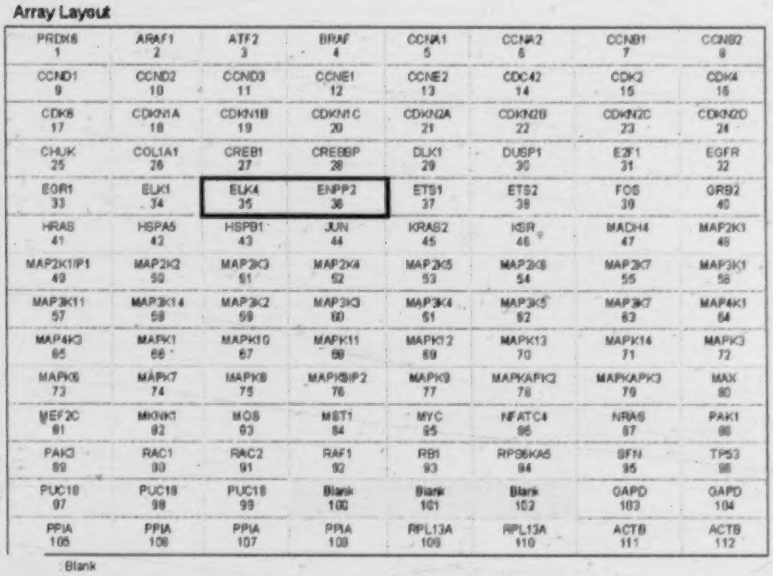


Figure 4.8.4 (I generated this figure)





Array Layout

PRDX6 1	ARAF1 2	ATF2 3	BRAF 4	CCNA1 5	CCNA2 6	CCNB1 7	CCNB2 8
CCND1 9	CCND2 10	CCND3 11	CCNE1 12	CCNE2 13	CDC42 14	CDK2 15	CDK4 16
CDK6 17	CDKN1A 18	CDKN1B 19	CDKN1C 20	CDKN2A 21	CDKN2B 22	CDKN2C 23	CDKN2D 24
CHUK 25	COL1A1 26	CREB1 27	CREBBP 28	DLK1 29	DUSP1 30	E2F1 31	EGFR 32
EGR1 33	ELK1 34	ELK4 35	ENPP2 36	ETS1 37	ETS2 38	FOS 39	GRB2 40
HRAS 41	HSPA5 42	HSPB1 43	JUN 44	KRAS2 45	KSR 46	MADH4 47	MAP2K1 48
MAP2K1IP1 49	MAP2K2 50	MAP2K3 51	MAP2K4 52	MAP2K5 53	MAP2K6 54	MAP2K7 55	MAP3K1 56
MAP3K11 57	MAP3K14 58	MAP3K2 59	MAP3K3 60	MAP3K4 61	MAP3K5 62	MAP3K7 63	MAP4K1 64
MAP4K3 65	MAPK1 66	MAPK10 67	MAPK11 68	MAPK12 69	MAPK13 70	MAPK14 71	MAPK3 72
MAPK6 73	MAPK7 74	MAPK8 75	MAPK8IP2 76	MAPK9 77	MAPKAPK2 78	MAPKAPK3 79	MAX 80
MEF2C 81	MKNK1 82	MOS 83	MST1 84	MYC 85	NFATC4 86	NRAS 87	PAK1 88
PAK3 89	RAC1 90	RAC2 91	RAF1 92	RB1 93	RPS6KA5 94	SFN 95	TP53 96
PUC18 97	PUC18 98	PUC18 99	Blank 100	Blank 101	Blank 102	GAPD 103	GAPD 104
PPIA 105	PPIA 106	PPIA 107	PPIA 108	RPL13A 109	RPL13A 110	ACTB 111	ACTB 112

Blank

Gene Name	*Fold increase*
Elk4/Sap 1a	3.05
autotaxin (ATX) (ENPP2)	4.75

Figure 4.8.5 (I generated this figure)

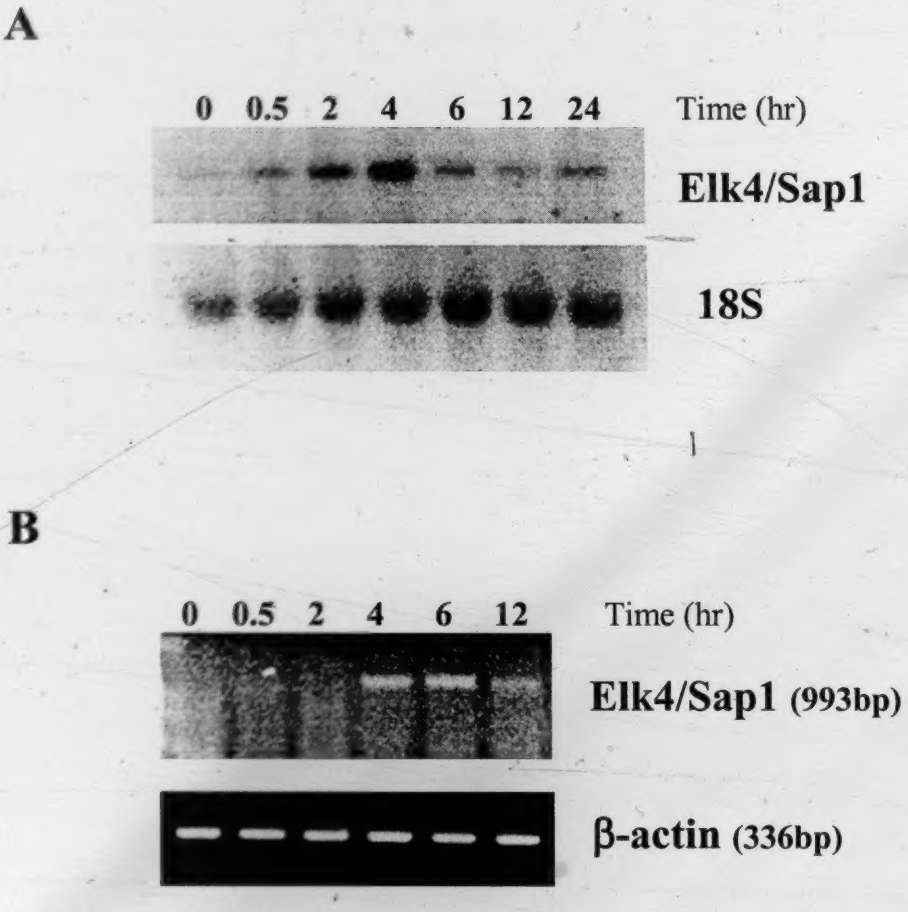

A
0
0.5
2
4
6
12
24
Time (hr)
Elk4/Sap1
18S
B
0
0.5
2
4
6
12
Time (hr)
Elk4/Sap1 (993bp)
β-actin (336bp)

Figure 4.8.6 (I generated this figure)

CHAPTER 5:

GENERAL DISCUSSION AND CONCLUSIONS

5.1 Discussion and Conclusions

The ability to generate or repair injured tissue is essential to the continuity of human life. Epidermal – mesenchymal interactions between keratinocytes and fibroblasts play a crucial role in modulating the expression of MMPs during development and healing of skin. Disruption of this interaction, as is the case with delays in epithelialization during the process of wound healing due to either infection or severity of injury, increases the frequency of developing fibrotic conditions.

In a previous study to identify MMP-1 stimulating factors produced by epidermis, Ghahary *et al* (2004) isolated a keratinocyte-derived factor from keratinocyte-conditioned medium and subsequently identified it as stratifin with potent MMP-1 stimulatory effects on dermal fibroblasts (1). No information concerning the regulation of this extracellular form of stratifin has been determined. Moreover, the mechanism by which stratifin induces MMP-1 in dermal fibroblasts is unknown. Therefore, this thesis represents an attempt to examine the regulation of stratifin's ability to increase MMP-1 and also the signal transduction pathway that mediates its actions.

In regards to examining the regulation of stratifin, I first observed that stratifin stimulation of MMP-1 mRNA expression was completely blocked by keratinocyte serum-free medium (KSFM). Since KSFM contains insulin, the effect of insulin on stratifin stimulation of MMP-1 expression was evaluated. The results documented that insulin played an important role in stratifin regulation of MMP-1 in dermal fibroblasts. The antagonistic effect of insulin on MMP-1 mRNA expression was time and dose dependent. The maximal antagonistic effect of insulin was seen at 36 hours post treatment. The conclusions of this study are that stratifin stimulates the expression of MMP-1 mRNA expression in dermal fibroblasts and this effect is antagonized by insulin treatment.

This finding may be relevant in chronic non-healing wounds among diabetic patients. Chronic wounds have been shown to possess increased MMP-1 levels and activity (2) as well as elevated MMP-1 transcripts in fibroblasts underlying the non-healing epithelium (3). Furthermore, MMP-1 protein levels are three-fold higher in chronic wound fluids than is observed for peak levels of fluids from healing dermal wounds (4). Indeed, non-healing wounds show evidence of excessive tissue destruction mediated by proteases. Impaired wound healing, such as in foot ulcers, is certainly correlated with the diabetic state (5-8). Several pathogenic mechanisms have been suggested to be involved in the development of diabetic skin complications; however, the exact molecular mechanism through which the level of MMP-1 remains high is not known. Our results show that insulin antagonizes stratifin-induced MMP-1 levels in fibroblasts; therefore, the absence of insulin production, as seen predominantly in type I insulin-dependent diabetes mellitus, may contribute ultimately to the elevated levels of MMP-1 observed in chronic diabetic wounds.

In general, MMPs are not constitutively expressed in skin but are induced temporarily in response to exogenous signals that elicit the proteolytic remodeling of the ECM (9). Previous studies have shown that MMP-1 gene expression is activated specifically via a promoter segment located between -95 to -72 bp upstream of the transcription initiation site, which contains adjacent binding sites for AP-1 and ETS transcription factors (10, 11). The expression and transactivation capacities of AP-1 and ETS transcription factors are regulated by MAPK pathways (12, 13). The results of my signal transduction studies reveal that stratifin induces fibroblast MMP-1 mRNA and protein levels and this is mediated by p38 MAP kinase. Moreover, my data show that treatment of dermal fibroblasts with stratifin resulted in rapid and transient up-regulation of *c-jun* and *c-fos*, which are components of AP-1. Microarray results revealed that stratifin-induced Sap1a may be involved in stratifin-induced *c-fos* transcription and activation, ultimately resulting in up-regulation of MMP-1 expression. In light of the findings, my data may support the use of pyridinylimidazole compounds that potently inhibit the enzymatic activity of p38 MAPKs, such as SB203580, in order to reduce the excessive MMP-1 environment seen in diabetic impaired wound healing.

My results that show stratifin to stimulate MMP-1 via a p38-dependent signaling pathway may be important for the study of tumor growth in light of the fact that MMP activity has been shown to be necessary for tumor invasion and metasis (14-20). An increase in MMP expression has been implicated with tissue degradation and remodeling during tumor invasion (21-23) and recent studies have revealed that MMPs may play a critical role in early events in tumour development (24-29). In addition, MMP-1 is expressed by several types of normal and malignant cells and the increased expression of this protease has been shown to correlate with invasiveness of malignant tumors including gastric and colon carcinomas (30, 31). It seems likely that signaling mechanisms by which tumors increase local MMP activity may constitute novel anti-tumor drug targets. Therefore, it is conceivable that specific inhibition of stratifin-induced p38 MAPK pathway may offer a novel therapeutic approach for reducing degradation of collagenous ECM as observed during tumor cell invasion.

Taken together these findings may provide insight into the mechanism by which insulin suppresses the MMP-1 stimulatory effect of stratifin. Studies have shown that PDGF suppresses MMP-1 gene expression via p38 MAPK (32). Although p38 may be the signal pathway by which insulin antagonizes MMP-1 expression in fibroblasts, studies have shown that p38 MAPK has bidirectional effect on MMP-1 expression (32). While p38 MAPK activation by IL-1 (33, 34), TNF-α (33), C2 cermaide (35), okadaic acid (36) upregulates MMP-1 gene expression, the opposite is observed when activation of p38 MAPK is induced by three-dimensional collagen lattices or arsenite resulting in the inhibition of MMP-1 gene expression (37, 38). To make matters even more complex, studies have reported that the effects of insulin on the p38 pathway are inconsistent. Insulin has been shown to stimulate p38 activity in cultured myoblasts involved in cytoskeletal rearrangement and myoblast differentiation (39, 40) as well as in L6 myotubes for insulin-induced glucose transport (41-44). Conversely, in fetal neurons, insulin inhibits the p38 pathway as well as apoptosis (45). Therefore, it is possible that insulin may antagonize MMP-1 mRNA expression via p38 MAPK pathway; however, whether the mode of action is by either activation or inhibition of the p38 pathway has yet to be made clear.

In conclusion, my results describe the mechanism by which stratifin activates MMP-1 in fibroblast as well as the antagonistic effects of insulin on the efficacy of stratifin. Taken these findings together, I demonstrate that the stratifin-induced *c-fos* and p38 MAPK activation are important regulators of MMP-1 gene expression involved in epidermal-mesenchymal interactions in wound remodeling.

5.2 Future Research

The results and conclusion presented in this thesis provide insight into the regulation and molecular mechanism of stratifin; however, several questions still remain.

While insulin is one of the components of KSFM and its effect partially blocked the stimulatory effect of stratifin, other remaining factors in KSFM may also contribute to the inhibitory effect in KSFM. Therefore, a new KSFM media was obtained in which supplements, such as insulin, hydrocortisone and transferrin, can be added separately. In the absence of each of these supplements, we evaluated the effect of KSFM on stratifin regulation of MMP-1. My preliminary results reveal that the efficacy of stratifin was restored nearly to completion when treatment was in KSFM without hydrocortisone (data not shown). This suggests that hydrocortisone could play a major role in the suppression of stratifin action. Subsequent experiments demonstrated the inhibitory effect of hydrocortisone on MMP-1 mRNA expression as dose-dependent. This finding is consistent with previous studies found in literature. Jonat et al (1999) demonstrated that glucocorticord hormone inhibits basal and phorbol ester-induced transcription of MMP-1 by interfering with AP-1, the major enhancer factor of the MMP-1 promoter (46). Various studies exhibit the mechanism of interference involve direct AP-1-glucocorticoid receptor interaction (46-49). Furthermore, Bauer et al (1985) showed glucocorticoid inhibition of MMP-1 expression in human skin fibroblast cultures (50). Continued experimentation is needed to confirm whether or not hydrocortisone is indeed an inhibitor of stratifin action.

My cDNA microarray analysis of fibroblasts treated with stratifin show an increase in a ternary complex factor, Sap-1, which binds to the serum response element of the *c-fos* promoter to stimulate *c-fos* transcription. However, another gene which stratifin modulates in dermal fibroblasts is autotaxin (ATX), a glycoprotein that belongs to the nucleotide pyrophosphatase and phosphodiesterase (NPP) family of ecto-enzymes (51). ATX possess lysophospholipase D (lyso-LPD) activity (52), which can generate the bioactive mediators lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P) (52, 53). Interestingly, studies have shown that LPA and S1P stimulate endothelial cell wound healing by increasing cell migration and proliferation (54). Moreover, both lipids are able to modulate the synthesis and release of peptide growth factors involved in wound healing. It has been demonstrated that LPA stimulates the secretion of TGF-β in keratinocytes (55) and insulin-like growth factor II in human breast and ovarian cancer cells (56, 57). Other documented biological effects of ATX include chemotaxis and angiogenesis thereby promoting wound healing through formation of new blood vessels (58). Ectopic expression of ATX can stimulate tumor progression, metasis and cell motility in an autocrine or paracrine manner (59). Furthermore, expression analysis has suggested a role for ATX in oligodendrocyte differentiation and myelination (60). Further studies are needed to evaluate the value of stratifin-induced ATX expression in the coordinated events of wound healing.

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